Ca^{2+}-DEPENDENT ASSOCIATION BETWEEN A Ca^{2+}-ACTIVATED NEUTRAL PROTEINASE (CaANP) AND ITS SPECIFIC INHIBITOR

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1. Introduction

A specific inhibitor of the calcium-activated neutral proteinase (CaANP) detected first in [1] was subsequently purified from rabbit skeletal muscle [2] and bovine cardiac muscle [3]. When we have purified CaANP from rabbit skeletal muscle, no calcium-stimulated proteolytic activity on casein could be detected in the crude extract but after separation from the inhibitor by ion-exchange chromatography, this activity was easily measured. The presence of a similar proteinase inhibitor was also demonstrated in various other tissues (brain, lung, heart, liver) [4–6]. The simultaneous presence of CaANP [5–10] in these tissues seems to indicate that proteolysis by this enzyme may be closely controlled. Ca^{2+} may play a role in the association between the enzyme and its specific inhibitor [3,5,11].

This work examines the effects of Ca^{2+} on the mechanism of the enzyme–inhibitor interactions. The results obtained show that the association is dependent on Ca^{2+} while chelating agents (EDTA or EGTA) induce a partial dissociation of the complex. The inhibitor does not bind to the enzyme through Ca^{2+} but to the enzyme–Ca^{2+} complex which represents the 'active form'.

2. Experimental procedure

2.1. *Purification of CaANP and inhibitor*

Fresh rabbit (Fauve de Bourgogne) skeletal muscle was homogenized in 2.5 vol. 5 mM Tris–HCl buffer (pH 7.40) containing 4 mM EDTA, 50 mM NaCl and 2 mM 2-mercaptoethanol as in [12] and used as the starting material for the preparation of CaANP and its inhibitor.

CaANP was made from the homogenate according to our standard procedure up to the S_{200} step [12]. Consequently, the enzyme still contained one 73 000 M_r and two 30 000 M_r polypeptide chains. Casein (Merck) was used as a substrate to quantitate proteolytic activity as in [12].

A separate portion of the homogenate was used for the preparation of the inhibitor by a modification of the method in [2]. After centrifugation (27 000 × *g* for 40 min) the supernatant was run on a column of DEAE–Sephacel (2.5 × 60 cm) equilibrated in 5 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM EDTA, 50 mM NaCl and 2 mM 2-mercaptoethanol. The inhibitor was then eluted from the column by a linear gradient of NaCl (50–500 mM) in the same buffer. The pooled inhibitor fractions were adjusted to 30% saturation ammonium sulfate solution by dialysis at 4°C. The insoluble material was removed by centrifugation (30 000 × *g* for 20 min) and the supernatant applied to a column of octyl-Sepharose (2.5 × 20 cm) equilibrated with a buffer containing 5 mM Tris–HCl (pH 7.40), 50 mM NaCl, 3 mM 2-mercaptoethanol. The inhibitor was eluted with the same buffer containing 10% saturated $(NH_4)_2SO_4$, the active fractions were pooled, dialysed overnight and then purified as before using organo-mercurial Sepharose and Ultrogel Ac A44 [2].

Inhibitor activity was determined by our standard assay [2].

2.2. *Immobilization of inhibitor and CaANP*

The inhibitor-conjugated Sepharose was prepared as described in Pharmacia Fine Chemical AB. Pure inhibitor (10 mg) was mixed with 1.5 mg pre-swollen CNBr-activated Sepharose. The binding efficiency of the protein to the matrix (evaluated using protein

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determination on inhibitory activity) was ~75%.

CaANP S_{200} (~15 mg) was adsorbed to DEAE-Sephacel column (1.5 × 10 cm) using Tris–HCl buffer (pH 7.40) containing 180 mM NaCl, 0.1 mM EDTA and 2 mM 2-mercaptoethanol.

The interaction of inhibitor, enzyme and activation was tested by measuring the proteolytic activity eluted from the column [2,12].

2.3. *Other methods*

Protein concentrations were determined by the modification [13] of the method in [14] using bovine serum albumin as standard. Analytical SDS gel electrophoresis was performed according to [12]. Ca^{2+} concentrations were estimated using a Pye Unicam atomic absorption spectrometer.

3. Results and discussion

3.1. *Binding of the CaANP to the CNBr-activated Sepharose–inhibitor column*

The involvement of Ca^{2+} in the association between enzyme and inhibitor was evaluated by measuring the binding of the enzyme to the column in presence or absence of Ca^{2+}.

Fig.1 shows that the amount of CaANP bound is a function of $[Ca^{2+}]$; 50% of the total bound was obtained with a 1.5 mM Ca^{2+} solution. Other ions like Mg^{2+} and Sr^{2+} used at the same concentrations had no effect on the association of enzyme and inhibitor even at 3 mM.

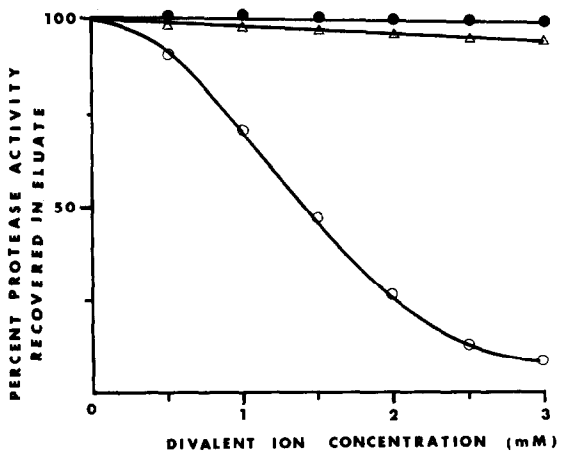

Fig.1. Effect of Ca^{2+}, Sr^{2+} and Mg^{2+} on the association enzyme–inhibitor-conjugated Sepharose. Aliquots of CaANP (250 μl ≃ 10 units) were applied with each concentration of divalent ions between 0–3 mM, on the inhibitor-conjugated Sepharose column (1.5 × 2 cm) equilibrated in 10 mM Tris–HCl (pH 7.40), 300 mM NaCl, 2 mM 2-mercaptoethanol. The column was eluted with a buffer containing (○——○) $CaCl_2$; (●——●) $SrCl_2$ or (△——△) $MgCl_2$. Aliquots (200 μl) of each column fraction were assayed for protease activity.

The enzyme–Ca^{2+}–inhibitor complex is dissociated by chelating agents like EDTA and EGTA. When EDTA and EGTA concentrations were increased from 0.5–3 mM, the percent of free enzyme recovered was also increased (fig.2) reaching ~50% of the activity loaded on to the column originally.

Electrophoretic analysis (fig.3) of the eluate from the inhibitor-conjugated Sepharose column shows that only the 73 000 M_r component is bound to the inhibitor in presence of calcium.

3.2. *Binding of the inhibitor to the immobilized DEAE-Sephacel CaANP*

During the preparation of inhibitor and enzyme [2], we have shown that these two molecules were both adsorbed by DEAE–Sephacel and eluted from the ion-exchange column at 0.1 and 0.35 M NaCl, respectively, when a linear gradient of ionic strength

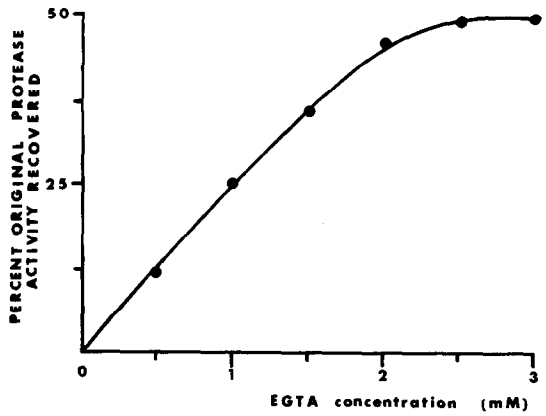

Fig.2. Effect of chelating agents on the dissociation of the enzyme–inhibitor complex. For each experiment, an aliquot of CaANP (250 μl ≃ 10 units) was applied on the inhibitor-conjugated Sepharose column (1.5 × 2 cm) equilibrated in 10 mM Tris–HCl (pH 7.40), 300 mM NaCl, 2 mM 2-mercaptoethanol and 3 mM $CaCl_2$. After washing with the equilibration buffer, the enzyme was eluted with 10 mM Tris–HCl (pH 7.40), 300 mM NaCl, 2 mM 2-mercaptoethanol and EGTA at one concentration (between 0.5–3 mM). Eluted column fractions were assayed and pooled for protease activity. After each experiment, the inhibitor-conjugated Sepharose was regenerated using a buffer containing 10 mM Tris–HCl (pH 7.40), 300 mM NaCl, 2 mM 2-mercaptoethanol and 3 mM EGTA.

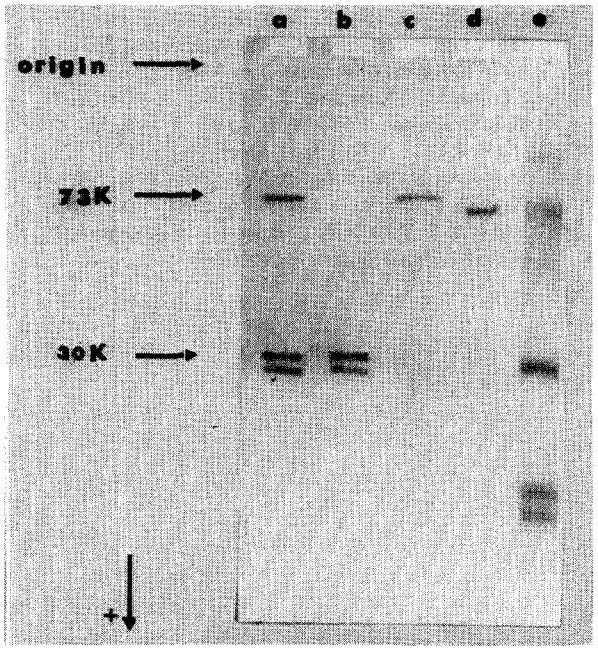

Fig.3. SDS–polyacrylamide gel electrophoresis before and after binding to the inhibitor–Sepharose 4B column: (a) CaANP (S_{200}) without calcium; (b) CaANP (S_{200}) with 3 mM calcium; (c) CaANP (S_{200}) bound to the inhibitor–Sepharose 4B column and eluted; (d) purified inhibitor as used in the coupling reaction to the CNBr-activated Sepharose 4B; (e) standard proteins (M_r): phosphorylase B, (98 000); bovine serum albumin (68 000); carbonic anhydrase (30 000); α-lactalbumin (14 000).

was applied. Between those two values of ionic strength only the enzyme is bound to the matrix. Even with a high concentration of Ca^{2+} (3 mM) in the buffer, the inhibitor was still eluted indicating that there was no complex formation on this support.

3.3. *Calcium binding to the inhibitor*

Unlike CaANP, the inhibitor is stable in solutions containing calcium. To study calcium binding by the inhibitor, experiments using equilibrium dialysis were performed in microcells. At various protein (1–3 mg in 2 ml) and calcium (0.2–3 mM) levels, no binding was detected in our experimental system.

4. Discussion

Most cells, including muscle cells contain both the CaANP and its specific inhibitor [4,9]. The proteinase is activated in vitro by calcium. Inhibition did not result from competition for Ca^{2+} by the inhibitor [2,15]. The results obtained here agree with those in [3,5,11] showing a Ca^{2+}-dependent regulation of CaANP. Therefore the inhibition mechanism can be explained by either of two hypotheses:

(1) Ca^{2+} provide the link between the enzyme and its inhibitor;
(2) Ca^{2+} bound by the enzyme induce a structural modification which promotes complex formation with the inhibitor.

Our results accord with the second hypothesis, since:

(i) The use of inhibitor-conjugated Sepharose has given direct proof that there is interaction between the enzyme and inhibitor in the presence of calcium;
(ii) Using equilibrium dialysis we have shown that the inhibitor does not bind calcium;
(iii) The adsorption of the enzyme on DEAE–Sephacel (which neutralises negative charges on the molecule) suppresses the interaction of the inhibitor, probably because the calcium can no longer bind electrostatically to the enzyme.

These conclusions are confirmed by the observations [11] that one inhibitor molecule could inactivate and link 10 enzyme molecules. Such an observation would be very unlikely if Ca^{2+} provided the linkage.

These experiments provide evidence for an indirect role of calcium in the association of CaANP with its inhibitor.

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