AUTOLYTIC AND PROTEOLYTIC PROCESSES OF CALCIUM-ACTIVATED NEUTRAL PROTEASE ARE INDEPENDENT FROM EACH OTHER

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ABSTRACT

To confirm the existence of an autolytic process, independent of the protease activity towards substrate, in the activation of calcium-activated neutral protease (CANP), differences in the mode of autolysis and proteolysis were examined. While the optimal pH for proteolysis by autodigested μ CANP is 7.2, the pH optimum for the autolysis by native μ CANP is higher than pH 8. The calcium ion-requirements for the two reactions are different, and the rate of autolysis increases rather than decreases in the presence of substrate.

Calcium-activated neutral protease (CANP) is an intracellular thiol protease which is activated by calcium ions. There are two kinds of CANP, namely μ CANP which is activated at μ M concentrations of calcium ions and mCANP for which nearly mM concentrations are required (9). Thus μ CANP can be active in the physiological state, whereas mCANP is active only under pathological conditions. Both CANPs are autolyzed in the presence of calcium ions, and the autolyzed species become more sensitive to calcium ions than the intact enzyme (1, 3, 14). Much evidence is being accumulated that the intact CANP is a proenzyme and must be autolyzed to obtain protease activity (11-13). If this is so, an autolysis process preceding the activation of CANP should exist. This process is also proteolytic as a matter of course. To establish the autolytic activation mechanism of CANP, it is necessary to show the existence of an autolytic proteolysis which is independent from the proteolysis of substrates by CANP. In this paper we will present some evidence to demonstrate that such a process exists.

MATERIALS AND METHODS

Materials

μ CANP and vimentin were purified from

rabbit skeletal muscle and mouse ascites hepatoma cells, respectively, as described previously (4, 5). Casein (nach Hammarsten) was obtained from Merck.

Autolysis of μ CANP

For the determination of the pH dependence of autolysis, native μ CANP was incubated in 0.1 M Tris-acetate buffer of various pHs at 30°C in the presence of 250 μ M CaCl_2 . After 1.5 min, aliquots were mixed with the sample buffer for electrophoresis. For the preparation of autodigested μ CANP, native μ CANP was autolyzed to a 76 K species in the presence of CaCl_2 and vimentin (5). Autolytic conversion of 79 K subunit to 77 K and 76 K subunits was followed by SDS-polyacrylamide gel electrophoresis according to Laemmli (8).

Determination of Protease Activity

CANP activity was measured using alkali-denatured casein as a substrate (7). For the determination of pH dependence, 0.1 M Tris-acetate buffer of various pHs and 250 μ M CaCl_2 were used. For the determination of calcium dependence, 0.1 M Tris-HCl buffer (pH 7.5) and calcium-EGTA buffer (2) were

Fig. 1 Effect of pH on the autolytic and proteolytic activities of μ CANP. A: Native μ CANP was incubated in 0.1 M Tris-acetate buffer of the indicated pH at 30°C for 1.5 min in the presence of 250 μ M CaCl_2 and the autolysis of 79 K subunit was followed by SDS-polyacrylamide gel electrophoresis. B: Native μ CANP (—●—) or autodigested μ CANP (---○---) was assayed for proteolytic activity at various pHs using casein as a substrate. For each enzyme, the highest activity attained was taken as 100%.

used.

RESULTS

Effect of pH

In order to find any difference between the autolysis of native μ CANP and the proteolysis of substrates by activated μ CANP, we first examined the pH dependence of these reactions. As seen in Fig. 1, the optimum for hydrolysis of the substrate by activated μ CANP is at pH 7.2 while the optimum for the autolysis of native μ CANP is higher than pH 8. Consistent with this finding, the hydrolysis of substrate by native μ CANP shows a broader pH dependence ranging to higher pH

than does the activated μ CANP. Thus, it is reasonable to conclude that the hydrolysis of substrate by native μ CANP consists of two sequential steps, namely the autolysis of native μ CANP to an activated protease and the hydrolysis of substrate by the activated CANP (6).

Effect of Substrate

If the autolysis is a side reaction of the proteolytic activity of native μ CANP, it should be inhibited competitively by substrate. However, the presence of substrate accelerates the conversion of the 79 K subunit to 76 K (Fig. 2). Moreover, the presence of substrate

Fig. 2 Effect of substrate concentration on the autolysis of μ CANP. Native μ CANP was incubated with $250 \mu\text{M}$ CaCl_2 in the presence of various concentrations of vimentin at 25°C for 2 min and the autolysis was followed by SDS-polyacrylamide gel electrophoresis. Lane 1, native μ CANP. For lanes 2–7, the concentrations of vimentin added were 0, 29, 57, 115, 229 and $459 \mu\text{g/ml}$ from left to right, respectively. (L), the large subunit of μ CANP

enhances the calcium ion sensitivity of the autolysis reaction (Fig. 3).

Calcium Dependence of Autolysis and Proteolysis

It is well-known that autolysis renders CANP more sensitive to calcium ions (1, 3, 14). In our experiment, half-maximal proteolytic activity of native μ CANP was attained at $30 \mu\text{M}$ calcium ions, a value that was reduced to $1.5 \mu\text{M}$ after autolysis (Fig. 4). This means that the catalytic activity of activated μ CANP requires calcium ion concentrations in the physiological range but that autolytic activation of native μ CANP requires much higher concentrations. Thus autolysis and proteolysis depend differently on calcium ions.

DISCUSSION

The results described above clearly demonstrate that the autolytic and proteolytic processes of μ CANP are independent from each other and that autolysis is an activation process prerequisite to the realization of protease activity towards substrates of intact μ CANP which is inactive as a protease (6). Many proteases are known to exist as zymogens and are proteolytically, in some cases autolytically, activated when necessary. CANP belongs to this group of proteases and is activated by autolysis triggered by calcium ions. Since the pH and calcium dependence of the autolytic and proteolytic activities are different, it is possible that there exist two catalytic sites in CANP, one for autolysis and another for pro-

Fig. 3 Effect of substrate on the calcium dependence of autodigestion of μ CANP. Native μ CANP was incubated with various concentrations of free calcium ions in calcium-EGTA buffer in the presence (lanes 1-9) or the absence (lanes 10-18) of 196 μ g/ml vimentin at 25°C for 2 min and the autolysis was followed by SDS-polyacrylamide gel electrophoresis. The free calcium ion concentrations were 0, 3, 6, 9, 20, 40, 60, 100 and 200 μ M for lanes 1 and 10, lanes 2 and 11, lanes 3 and 12, lanes 4 and 13, lanes 5 and 14, lanes 6 and 15, lanes 7 and 16, lanes 8 and 17, and lanes 9 and 18

Fig. 4 Effect of calcium ion concentration on the activity of native and autodigested μ CANPs. Native μ CANP (1.16 units, —●—) or autodigested μ CANP (0.86 units, —○—) was assayed for protease activity using alkali-denatured casein as a substrate at various concentrations of free calcium ions.

teolysis. However, since no protease domain other than a thiol protease domain has been found in the primary structure of CANP (10), it seems likely that the two reactions share a common active site. We have examined the effect of protease inhibitors on these two reactions. The CANP inhibitors such as leupeptin, E-64c and moniodoacetic acid, however, blocked both the autolysis and proteolysis activities (data not shown). Thus, there is no evidence at present to indicate the presence of two active sites in CANP and it is reasonable to conclude that the two reactions are catalyzed at one active site. Our speculation is that the active site is buried in the interior of the native CANP molecule and that it catalyzes an intramolecular autolysis in the presence of calcium ions. After autolysis, the active site becomes exposed to the outside and can interact with exogenous substrates. High pH as well as high calcium ion concentrations are required to activate the site in native CANP, but the activated site requires low calcium ion concentrations and functions at neutral pH. The effect of substrate on autolysis also suggests that it is an intramolecular process analogous to the activation of pepsinogen to pepsin.

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