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Cytosolic Ca^{2+}-dependent neutral proteinases from rabbit liver: Activation of the proenzymes by Ca^{2+} and substrate

(denatured globin/casein)

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ABSTRACT **Two neutral Ca^{2+}-dependent proteinases, differing in molecular size, have been isolated from rabbit liver. Both are recovered as inactive proenzymes that can be converted to the active forms by high (0.1–1.0 mM) concentrations of Ca^{2+} in the absence of substrate or, in the presence of a protein substrate, by low (1–5 μM) concentrations of Ca^{2+}. The activated proteinases required only 1–5 μM Ca^{2+} for maximal activity. Substrates hydrolyzed were denatured globin, globin, casein, and to a lesser extent, several extracellular proteins; no digestion was observed with several intracellular cytosolic enzymes tested. Only those proteins that served as substrates were capable of promoting conversion of the proenzymes to the active low-Ca^{2+}-requiring proteinases.**

Ca^{2+}-activated neutral proteinases have been the subject of increasing interest since the suggestion by Huston and Krebs in 1968 (1) that phosphorylase b kinase activating factor is such a proteinase and more recent evidence for the role of Ca^{2+}-activated proteinases in the turnover of muscle proteins (ref. 2 and references therein). Ca^{2+}-requiring proteinases have also been implicated in the activation of a protein kinase of rat brain (3). Purified preparations have been described from muscles of a variety of species (2, 4–9), from human erythrocytes (10), and from rat liver (11–13) and other tissues (13).

The Ca^{2+}-activated proteinases from muscle have been reported to fall into two classes on the basis of their requirement for millimolar or micromolar concentrations of Ca^{2+} (refs. 5 and 9; for review see ref. 14) and similar high-Ca^{2+}- and low-Ca^{2+}-requiring proteinases have been identified in rat liver (11, 12). Although several laboratories have reported the conversion of high-Ca^{2+}-requiring proteinases to low-Ca^{2+}-requiring forms by limited autolysis (7, 15, 16), others have reported the isolation of high-Ca^{2+}- and low-Ca^{2+}-requiring forms that do not appear to be structurally related (9, 11).

In the present report we describe two low-Ca^{2+}-requiring proteinases from rabbit liver. These are isolated as inactive proenzymes that can be converted to the active forms by high concentrations of Ca^{2+} or, more physiologically, by low concentrations of Ca^{2+} in the presence of a digestible substrate.

MATERIALS AND METHODS

Materials. Farm-grown adult rabbits weighing 2.0–2.5 kg were purchased on the local market. Human acid-denatured globin was prepared as described by Hayman and Alberty (17) and extensively dialyzed against distilled water before use. Casein (type I), muscle aldolase (grade I), and bovine serum albumin (crystallized, lyophilized) were purchased from Sigma. Rabbit liver fructose-1,6-bisphosphatase was purified as described (18). Ultrogel AcA 34 and DEAE-Trisacryl M were purchased from LKB, Sephadex G-200 from Pharmacia, butylagarose from Miles, and DEAE-cellulose (DE 32) from Whatman.

Methods. *Assay of the neutral proteinase activity.* Neutral proteinase activity was assayed with acid-denatured globin as substrate, measuring the liberation of free α-amino groups. The reaction mixture contained, in a final volume of 0.6 ml, 50 mM sodium borate buffer at pH 7.5, 1.2 mg of substrate, and $CaCl_2$ at the concentrations indicated. The reaction was started by the addition of the appropriate amount of enzyme and the mixture was incubated for 10 min at 20°C. After the addition of trichloroacetic acid (7.5% final concentration), the protein was removed by centrifugation and the free amino groups in an aliquot of the supernatant solution were determined with fluorescamine (19). The unit of enzyme activity was defined as the amount required to release 1 μmol of free amino groups in 10 min under these conditions.

Purification of the proenzymes. Homogenates prepared in 0.25 M sucrose containing 1.0 mM EGTA as described (20) were centrifuged for 20 min at 20,000 × *g* and the supernatant solution was centrifuged again for 45 min at 100,000 × *g*. The supernatant solution from 40 g of rabbit liver (190 ml) was concentrated to 10 ml by ultrafiltration on an Amicon YM-10 membrane. This solution was then chromatographed on an Ultrogel AcA 34 column, which yielded three peaks of neutral proteinase activity, corresponding to molecular masses of >400 kilodaltons (kDa), 200 kDa, and 150 kDa, respectively. The proteinases in the last two peaks required Ca^{2+} and accounted for 18% and 75%, respectively, of the total activity (Fig. 1).

The 150-kDa proteinase was further purified by chromatography on butylagarose and Sephadex G-200. The 200-kDa proteinase was further purified by chromatography on DE 32, DEAE-Trisacryl M, and Sephadex G-200. The details of the purification procedures will be described elsewhere. The specific activities of the purified 150- and 200-kDa proteinases were 34.6 and 23.5 units/mg, respectively.

RESULTS

Conversion of the Proenzymes to the Active Low-Ca^{2+}-Requiring Proteinases. The effect of Ca^{2+} concentration on the rate of conversion of the 150- and 200-kDa proenzymes to the active low-Ca^{2+}-requiring forms is illustrated in Fig. 2. For the 150-kDa proteinase the rate in the presence of 1 mM Ca^{2+} was too fast to measure (Fig. 2*A*); in the presence of 100 μM Ca^{2+} the same conversion required several minutes (Fig. 2*B*). Similar conversions, although at somewhat lower rates, were observed with the 200-kDa proteinase (Fig. 2 *D* and *E*). These results resemble those reported by Suzuki and coworkers for the Ca^{2+}-requiring neutral proteinases from chicken muscle (15, 16). In contrast to the reports from other laboratories, however, we also observed a slower, but highly significant, rate of activation of the rabbit liver proen-

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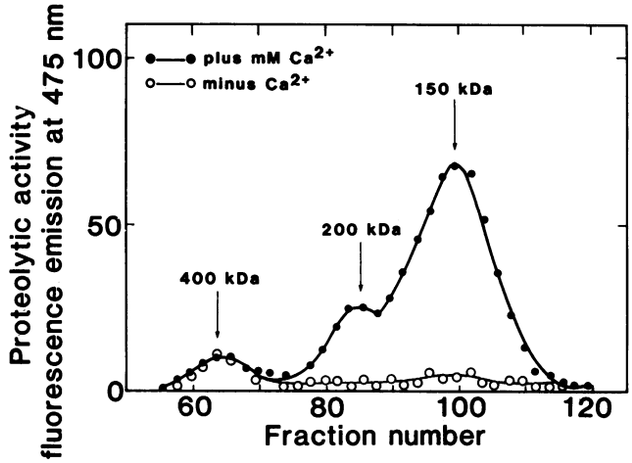

FIG. 1. Separation on Ultrogel AcA 34 of the neutral proteinase activities present in rabbit liver cytosol. The concentrated liver cytosol was prepared as described in the text and applied to a column (2.2 × 160 cm) of Ultrogel AcA 34 equilibrated in 50 mM sodium borate, pH 7.5, containing 0.1 mM EGTA and developed with the same buffer at a flow rate of 18 ml/hr . Fractions (3.5 ml) were collected and 100-μl aliquots were assayed in the absence (○) or presence (●) of 1 mM Ca^{2+}. The 150-kDa proteinase was then purified from fractions 95–115 as outlined in *Materials and Methods*. The 200-kDa proteinase was purified from the cytosol fraction by chromatography on a DE 32 column, which did not retain the 150-kDa proteinase, followed by chromatography on DEAE-Trisacryl M and Sephadex G-200.

zymes by a physiological (5 μM) concentration of Ca^{2+} (Fig. 2 *C* and *F*). In a similar experiment with 1 μM Ca^{2+} the conversion of the 150-kDa proteinase reached 40–50% of maximum in 3 hr (data not shown).

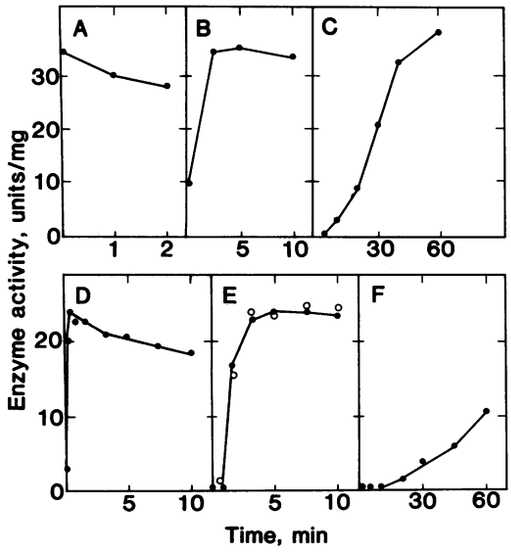

FIG. 2. Conversion of the native proenzymes to the low-Ca^{2+}-requiring forms. The purified 150-kDa proteinase (200 μl, 30 μg) was diluted to 2.0 ml with 50 mM sodium borate, pH 7.5, containing 4 mg of denatured globin and 1 mM Ca^{2+} (*A*), 100 μM Ca^{2+} (*B*), or 5 μM Ca^{2+} (*C*). The solutions were incubated at room temperature. At the times indicated 200-μl aliquots were treated with sufficient EGTA to reduce the Ca^{2+} concentration to approximately 5 μM and assayed in the presence of 5 μM Ca^{2+}. The purified 200-kDa proteinase (500 μl, 38 μg) was similarly incubated in the presence of 4 mg of denatured globin with 1 mM (*D*), 100 μM (*E*), or 5 μM (*F*) Ca^{2+}. Aliquots were assayed for neutral proteinase activity in the presence of 5 μM Ca^{2+}. In *E*, aliquots were also assayed in the presence of 100 μM Ca^{2+} (○).

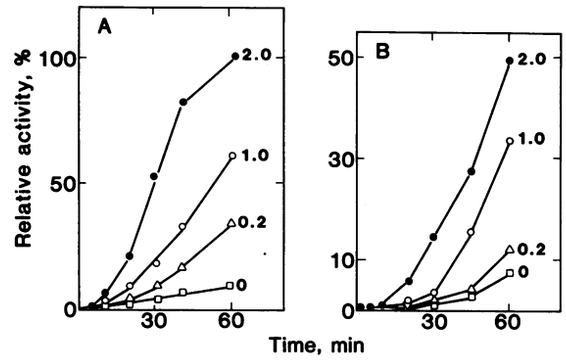

FIG. 3. Effect of substrate concentration on the activation of the proenzyme in the presence of 5 μM Ca^{2+}. (*A*) The 150-kDa proteinase (200 μl, 30 μg) was incubated in the presence of 5 μM Ca^{2+} as described in the legend to Fig. 2, with the mg amounts of denatured globin indicated in the figure. (*B*) The 200-kDa proteinase (500 μl, 38 μg) was incubated and assayed as described in *A* for the 150-kDa proteinase. In each case the activity measured directly in 1 mM Ca^{2+} was taken as 100%. These values were 32.5 and 21.3 units/mg of protein for the 150- and 200-kDa proteinases, respectively.

These observations suggested that the neutral proteinases from rabbit liver are indeed low-Ca^{2+}-requiring proteinases but that an obligatory first step is the conversion of the inactive proenzyme to the active enzyme forms. Once activated, these enzymes were found to be almost equally active with 100 μM or 5 μM Ca^{2+} (illustrated in Fig. 2*E* for the 200-kDa proteinase) (see also Fig. 4).

Effect of Substrate on the Conversion of the Proenzymes to the Low-Ca^{2+}-Requiring Forms. In the preceding experiments the globin substrate was always present when the enzymes were incubated with Ca^{2+}. On examination, the substrate was found to have no effect on the activation of either the 150-kDa or the 200-kDa proenzyme in the presence of 1 mM or 100 μM Ca^{2+} (data not shown) but to be essential for activation in the presence of 5 μM Ca^{2+} (Fig. 3). The rate of activation increased with increasing substrate concentration and was more rapid with the 150-kDa proenzyme. It also increased as the proportion of active-low-Ca^{2+}-requiring proteinases increased, suggesting that it was this active species that was responsible for the autocatalytic conversion.

Ca^{2+} Requirement for the Activated Neutral Proteinases. The activated proteinases required only 5 μM Ca^{2+} for maximal rates of hydrolysis of denatured globin (Fig. 4). Under the standard assay conditions, the proenzymes showed sig-

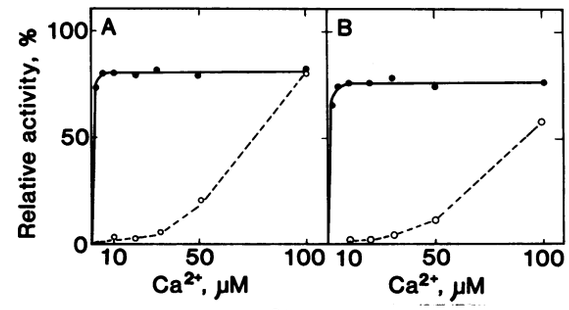

FIG. 4. Requirement of Ca^{2+} for the activity of the activated neutral proteinase and for expression of proenzyme activity. (*A*) The 150-kDa proteinase was activated in the presence of 5 μM Ca^{2+} and denatured globin at 2 mg/ml as described in the legend to Fig. 2. After 60 min at room temperature, aliquots (200 μl) were analyzed for neutral proteinase activity in the presence of the indicated concentrations of Ca^{2+} (●). The ○ show the activity of the proenzyme assayed directly without prior incubation with substrate and Ca^{2+}. (*B*) The same experiment was carried out with the 200-kDa proteinase. The values for 100% activity are as given in the legend to Fig. 3.

Table 1. Substrate specificity of the neutral 150-kDa proteinase

Substrate	Relative activity
Denatured globin	100
Native β-globin	16
Casein	15
Glucagon	7
Insulin B chain	4
Native human hemoglobin	2
Bovine serum albumin	<1
Rabbit liver fructose-1,6-bisphosphatase	<1
Rabbit muscle aldolase	<1

The substrates were tested at concentrations of 2 mg/ml and 30 μg of 150-kDa proenzyme in the presence of 1 mM Ca^{2+}. With this concentration of Ca^{2+} the proenzyme is activated almost instantaneously. Denatured globin was prepared as described in *Materials and Methods*. β-Globin (21) and rabbit liver fructose-1,6-bisphosphatase (18) were prepared as described. Other proteins tested were from Sigma.

nificant activity only at concentrations of Ca^{2+} above 30 μM. However, this activity can be attributed to the conversion of the proenzymes to the activated proteinases; this conversion was sufficiently rapid at the high Ca^{2+} concentrations to account for the proteolytic activity detected during the standard 10-min incubation period.

Substrate Specificity for Activity and for the Conversion of Proenzymes at Low Concentrations of Ca^{2+}. The activated 150-kDa proteinase was most active with denatured globin but showed some activity toward several other exogenous proteins, such as native β-globin and casein (Table 1). Similar specificity was observed for the 200-kDa proteinase. No activity was observed with native muscle aldolase or native liver fructose-1,6-bisphosphatase as substrates.

Only those proteins that were digested by the activated proteinases were active in promoting the conversion of the proenzymes to the active proteinases (Fig. 5). In the presence of 5 μM Ca^{2+}, formation of the active low-Ca^{2+}-requiring proteinase was most rapid in the presence of denatured globin. With casein, which was hydrolyzed only 15% as rapidly as denatured globin, the rate of activation was only about one-fifth as rapid. Rabbit muscle aldolase and fructose-1,6-bisphosphatase, which were inactive as substrates, failed to promote the proenzyme-to-active enzyme conversion.

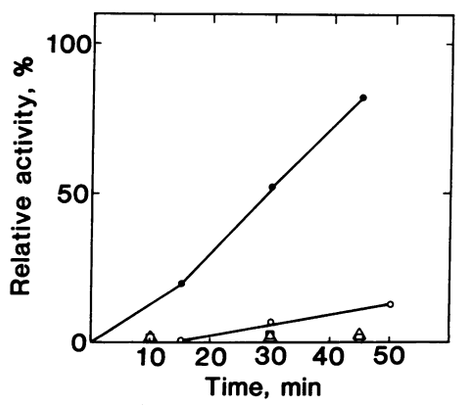

FIG. 5. Activity of substrate and nonsubstrate proteins as promoters of the proenzyme–active proteinase conversion in the presence of 5 μM Ca^{2+}. The conditions for activation of the proenzyme were as described in the legend to Fig. 4, with 15 μg of the purified 150-kDa proenzyme and 2 mg of proteins as indicated. Aliquots (200 μl) were assayed at the times indicated for activity with denatured globin as substrate, in the presence of 5 μM Ca^{2+}. The activity of the proenzyme assayed directly with 1 mM Ca^{2+} was taken as 100%. The proteins tested were denatured globin (●), casein (○), muscle aldolase (□), and bovine serum albumin (△).

DISCUSSION

The neutral proteinases of rabbit liver appear to be present in the cytosol in the form of inactive proenzymes that can be converted to the active low-Ca^{2+}-requiring forms by high concentrations of Ca^{2+} or, more physiologically, by low concentrations of Ca^{2+} in the presence of an appropriate digestible substrate. The finding that this conversion is almost instantaneous in the presence of 0.1–1.0 mM Ca^{2+} might lead one to conclude that the native forms, referred to here as proenzymes, are high-Ca^{2+}-requiring proteinases. However, the activated enzymes must be classified as low-Ca^{2+}-requiring proteinases, since they are equally active with 5 μM or 1 mM Ca^{2+}. The activity observed when the proenzymes are assayed in the presence of millimolar concentrations of Ca^{2+} can be attributed to the formation of active enzyme during the time required for the assay.

With physiological concentrations of Ca^{2+} (1–5 μM) the formation of active proteinase requires the presence of a digestible substrate. Only denatured or "foreign" proteins appear to be suitable substrates and activators, which may account for the fact that these proteinases are present in normal liver cytosol as the inactive proenzyme forms. This observation provides an attractive model for the regulation of the activity of these proteinases, although the physiological substrate(s) and activator(s) remain to be identified.

The function of the Ca^{2+}-requiring neutral proteinases in liver remains to be determined. In addition to a possible role in the activation of phosphorylase b kinase, which has not yet been demonstrated for the liver kinase, or in the activation of the proenzyme of the cAMP-independent protein kinase (3), Ca^{2+}-requiring proteinases have also been shown to hydrolyze the receptors for epidermal growth factor (22) and calmodulin-binding proteins (23). They may prove to play a major role in the regulation of these systems.

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