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Activation of calcineurin by limited proteolysis

(calmodulin/Ca^{2+}/protein phosphatase)

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ABSTRACT **Calcineurin, a heterodimer of calcineurin B, a 19,000 M_r Ca^{2+}-binding subunit, and calcineurin A, a 61,000 M_r calmodulin-binding subunit, was previously proposed to be a calmodulin- and Ca^{2+}-regulated protein phosphatase. Like other calmodulin-stimulated enzymes, calcineurin can be activated and rendered calmodulin- and Ca^{2+}-independent by limited proteolysis. By glycerol gradient centrifugation, the native enzyme has a $s_{20,w}$ of 4.5 S in EGTA and 5 S in the presence of Ca^{2+}·calmodulin. Under the same conditions, the $s_{20,w}$ of the trypsin-activated enzyme (4.3 S) is not affected by Ca^{2+} and calmodulin. The trypsin-treated enzyme is a heterodimer of calcineurin B and a 45,000 M_r fragment of calcineurin A that has lost its ability to interact with calmodulin. Phosphatase activity sediments with calcineurin or its proteolytic fragments, providing further evidence that calcineurin is indeed a protein phosphatase. Calmodulin protects calcineurin against tryptic digestion; proteolysis occurs more slowly, yielding fragments with M_r 57,000, 55,000, and 54,000 that have preserved their ability to interact with calmodulin. After trypsin treatment in the presence of calmodulin, the protein phosphatase activity of calcineurin is still regulated by calmodulin. Prolonged trypsin treatment in the presence of calmodulin produces a 46,000 M_r fragment. Unlike the fragments generated in the absence of calmodulin, this 46,000 M_r fragment still interacts weakly with calmodulin. Thus, calcineurin, like other calmodulin-regulated enzymes, consists of a catalytic domain resistant to proteolysis and a calmodulin-binding regulatory domain susceptible to protease action in the absence of calmodulin but not in its presence. In the absence of calmodulin, the regulatory domain exerts an inhibitory effect on the catalytic domain; the inhibition is relieved upon calmodulin binding to or tryptic degradation of the regulatory domain.**

Calcineurin, a major calmodulin-binding protein of brain (1–4), is a heterodimer composed of a 61,000 M_r calmodulin-binding subunit (calcineurin A) and a 19,000 M_r subunit (calcineurin B) capable of binding four Ca^{2+} (2). Recently, a Ca^{2+}- and calmodulin-regulated phosphoprotein phosphatase activity has been found to be associated with calcineurin (5, 6). Other calmodulin-stimulated protein phosphatases, the "protein phosphatases 2B," have been detected in a large number of tissues (7). The skeletal muscle enzyme, which has been purified to homogeneity, has a subunit composition similar to that of calcineurin (8). A calcineurin-like protein is also present at low levels in heart (9, 10). Whereas calcineurin and the calmodulin-regulated protein phosphatases from different tissues share similar enzymatic activities, they exhibit differences in the molecular weight of their large subunits. They may also differ antigenically, because calcineurin antibody appears to be relatively specific for the brain protein (11).

Calcineurin dephosphorylates several substrates, including smooth and skeletal muscle myosin light chains, phosphatase inhibitor 1, the α subunit of skeletal muscle phosphorylase kinase, and the type II regulatory subunit of cAMP-dependent protein kinase. Histones IIa and VS, and the β subunit of phosphorylase kinase are only poor substrates (7). The phosphatase activity is stimulated slightly by Ca^{2+} and is activated a further 2- to 4-fold by calmodulin (5, 6). It has been suggested that calcineurin A is the catalytic subunit, subject to regulation by two different Ca^{2+}-binding proteins, calcineurin B and calmodulin, acting at two distinct sites (5). In the present study, we show that limited proteolysis of calcineurin results in activation of the phosphoprotein phosphatase, with attendant loss of its calmodulin and Ca^{2+} stimulation. These functional changes are correlated with a decrease in the molecular weight of calcineurin A and loss of ability to bind calmodulin. In contrast, the Ca^{2+}-independent interaction between calcineurin A and B is not altered by limited proteolysis, confirming the existence of distinct sites on calcineurin A for interaction with calcineurin B and calmodulin.

MATERIALS AND METHODS

Calcineurin, purified from bovine brain as described (6), was dialyzed overnight at 4°C against 0.04 M Tris·HCl, pH 7.5, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 0.2 mM dithiothreitol in the presence or absence of 5% (vol/vol) glycerol prior to tryptic digestion or enzymatic assay. Calmodulin was purified from bovine testes by the method of Autric *et al.* (12) with modifications (unpublished results). ^{125}I-Labeled calmodulin (^{125}I-calmodulin) (specific activity 100 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was prepared as described (6). Dephosphorylated smooth muscle myosin light chains, prepared from chicken gizzard by the method of Perrie and Perry (13) with modifications (D. R. Hathaway and J. R. Haeberle, personal communication), and myosin kinase, prepared as described (14), were generously provided by David R. Hathaway (Indiana University School of Medicine). [^{32}P]Phosphorylated myosin light chains (specific activity 1,000 cpm/pmol) were prepared as described (6). [^{14}C]Guanidinated calmodulin (specific activity 200,000 cpm/nmol), prepared by the method of Klee and Richards (15), contained 1–2 mol of homoarginine per mol.

Limited tryptic digestion was performed by incubating calcineurin (0.1 mg/ml) with trypsin [treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Worthington), dissolved in 0.001 M HCl] at 0.35 μg/ml in the presence of 0.5 mM $CaCl_2$ at 30°C for the indicated times. When calmodulin (3 μM) was included in the digestion mixture, trypsin was increased to 1 μg/ml. Reactions were stopped by addition of soybean trypsin inhibitor at 100 μg/ml and reaction mixtures were cooled on ice.

Phosphatase activity was assayed in 50 μl of 0.02 M Tris·HCl buffer, pH 8.0, containing 0.1 M NaCl, 6 mM $MgCl_2$, bovine serum albumin at 0.1 mg/ml, 10 μM $MnCl_2$, 0.5 mM dithiothreitol, 0.3–0.8 μg of calcineurin, and 1 μM [^{32}P]phosphorylated myosin light chains (10^6 cpm/nmol). Calmodulin (1 μM),

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$CaCl_2$ (1 mM),or EGTA (0.2 mM) was included in assay mixtures as indicated. Incubations were conducted at 30°C for 5–10 min. Reactions were stopped by the addition of 0.5 ml of stop solution, prepared by 10-fold dilution of 1 M potassium phosphate, pH 7.0, with 5% trichloroacetic acid. Inorganic phosphate was isolated by passage through 0.5-ml columns of Dowex AG 50W-X8, H^+ form, 200–400 mesh, that had been washed sequentially with 10 ml water, 1 ml of 1 M NaOH, 2 ml of 1 M HCl, and 4 ml of water. Mixtures were applied to columns and eluates were collected directly in scintillation vials. After a rinse with 0.5 ml of water to ensure complete recovery of phosphate, 12 ml of Aquasol was added to each vial, and radioactivity was quantitated by liquid scintillation counting.

Glycerol Gradient Centrifugation. Aliquots (30–40 μg in 0.1 ml) of calcineurin or trypsin-treated calcineurin were layered on top of 10–40% (vol/vol) glycerol gradients (3.7 ml) resting on a 0.1 ml cushion of 75% glycerol. Gradients contained 0.05 M Tris·HCl at pH 8, 0.1 M NaCl, 3 mM $MgCl_2$, 0.1 mM dithiothreitol, soybean trypsin inhibitor at 2 μg/ml; and either 1 mM EGTA or 0.2 mM $CaCl_2$ and 0.75 μM [^{14}C]guanidinated calmodulin. Centrifugation was performed in a Beckman L5-65 centrifuge at 55,000 rpm at 2°C for 20 hr in an SW 60 rotor. At the conclusion of the run, 6-drop (0.13-ml) fractions were collected from the bottom by pumping through a 20-μl pipet (Clay–Adams) inserted through the top of the tubes. Recoveries of phosphatase activity were 60–80%. Sedimentation coefficients were calculated by linear interpolation between lactate dehydrogenase ($s_{20,w}$ = 7.6 S) (16), bovine serum albumin ($s_{20,w}$ = 4.46 S) (17), and β-lactoglobulin ($s_{20,w}$ = 3.12 S) (17).

Gel electrophoresis in the presence of $NaDodSO_4$ was carried out in slabs of 7–15% gradients of acrylamide with the Laemmli system (1, 18). Molecular weight standards were phosphorylase *a* (97,000), bovine serum albumin (68,000), catalase (58,000), fumarase (48,000), actin (42,000), lactate dehydrogenase (35,000), and β-lactoglobulin (17,500). The subunits of calcineurin were quantitated by densitometric analysis of photographs of the Coomassie blue-stained gels, using a soft laser scanning densitometer (LKB). The color values of the different subunits were assumed to be proportional to their molecular weights, 61,000 and 19,000. [^{14}C]Guanidinated calmodulin was quantitated by liquid scintillation counting. ^{125}I-Calmodulin gel overlay was performed by the method of Carlin *et al.* (19) with minor modifications as described (20).

RESULTS

Activation of Calcineurin by Limited Proteolysis. The effect of limited proteolysis of calcineurin on the Ca^{2+}- and calmodulin-regulated protein phosphatase has strengthened the identification of calcineurin as a protein phosphatase and clarified the role of subunit interactions in the regulation of the enzyme. Limited tryptic digestion of calcineurin was performed in the absence or presence of 3 μM calmodulin. Effects of limited proteolysis on phosphatase activity are presented in Fig. 1. In the absence of calmodulin, treatment of calcineurin with trypsin for 4 min at 30°C resulted in activation of the enzyme assayed in the presence of EGTA, with concomitant loss of stimulation by Ca^{2+} and calmodulin (Fig. 1 *Left*). Phosphatase activity assayed in the presence of EGTA increased even further as a result of longer periods of trypsin treatment. In contrast, phosphatase activity assayed in the presence of Ca^{2+} and calmodulin was unaltered throughout 40 min of trypsin treatment (Fig. 1 *Left*).

When calmodulin was present during the proteolytic digestion, even though the protease concentration was increased 3-fold, phosphatase activity assayed in the presence of EGTA increased very slowly (Fig. 1 *Right*). Loss of enzyme stimulation by Ca^{2+} and calmodulin was observed only after 40 min of incubation. Phosphatase activity measured in the presence of Ca^{2+} and calmodulin was only minimally decreased after 40 min of digestion (Fig. 1 *Right*).

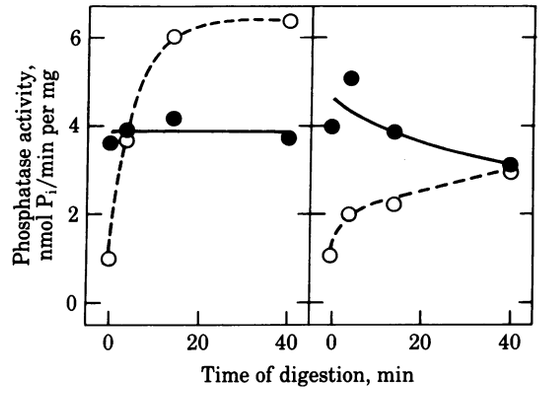

FIG. 1. Effects of limited proteolysis on regulation of the phosphatase activity of calcineurin. Limited tryptic digestion of calcineurin was conducted in the absence (*Left*) or presence (*Right*) of 3 μM calmodulin. Trypsin concentration was 0.35 μg/ml (*Left*) or 1.0 μg/ml (*Right*) (see text). Phosphatase activity was then assayed in the presence of either 0.2 mM EGTA (○) or 1 mM $CaCl_2$ and 1 μM calmodulin (●).

The course of proteolysis was also monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis, Coomassie blue staining, and ^{125}I-calmodulin binding to the gel. These methods provided an estimate of the size of the digestion products and assessment of their ability to interact with calmodulin. At zero time, the pattern of Coomassie blue staining revealed calcineurin A (M_r, 61,000), calcineurin B (apparent M_r, 15,000), and soybean trypsin inhibitor (M_r, 20,000) (Fig. 2 *Left*, lane labeled 0). The corresponding autoradiogram revealed ^{125}I-calmodulin labeling of calcineurin A (Fig. 2 *Right*, lane labeled 0). In the absence of calmodulin, incubation of calcineurin with trypsin for 4 min resulted in an 80% decrease in the band corresponding to calcineurin A, with appearance of a fragment of apparent M_r 45,000. Labeling of intact calcineurin A by ^{125}I-calmodulin was proportionately reduced. The M_r 45,000 fragment was not labeled by ^{125}I-calmodulin. As the digestion proceeded, intact calcineurin A disappeared, and the M_r 45,000 fragment accumulated. A further gradual conversion to slightly smaller species (M_r, 44,000 and 40,000) also occurred. None of these fragments was labeled with ^{125}I-calmodulin. Calcineurin B was not detectably altered by trypsin treatment under these conditions. No small peptides were detected by Coomassie blue staining or ^{125}I-calmodulin gel overlay.

The presence of calmodulin reduced the susceptibility of calcineurin to tryptic digestion (Fig. 2 *Left*). The rate of disappearance of calcineurin A was slowed, and the pattern of digestion was altered. A fragment of M_r 57,000 was detected 4 min after addition of trypsin. Upon further digestion, smaller fragments with M_r 55,000, 54,000, 46,000, and 40,000 were generated. Each of the four largest fragments bound calmodulin. At 40 min of digestion, a faint labeling was also detected at M_r 46,000. Again, no change in calcineurin B was observed throughout the digestion.

Effect of Limited Proteolysis on Calcineurin Subunit Interactions. The ability of calcineurin and its proteolytic products (prepared by incubation with trypsin for 40 min in the absence of calmodulin) to interact with calmodulin was followed by glycerol gradient centrifugation in the presence of Ca^{2+} and [^{14}C]-guanidinated calmodulin as illustrated in Fig. 3. Calcineurin A and B, measured by densitometric analysis of $NaDodSO_4$ gel

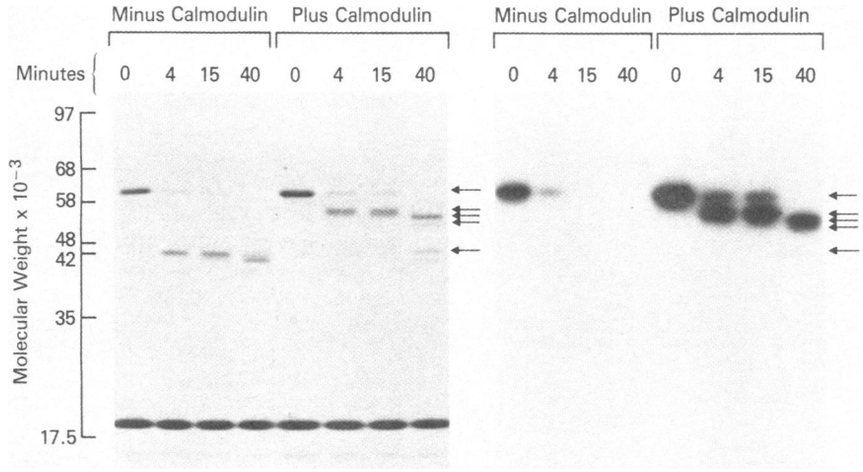

FIG. 2. Effect of limited proteolysis of calcineurin on subunit size and ability to interact with calmodulin. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed on 25-μl aliquots of the material tested for phosphatase activity as shown in Fig. 1. The slab gel was subjected to ^{125}I-calmodulin gel overlay. (*Left*) Coomassie blue stained gel, (*Right*) autoradiogram. Arrows denote bands that were labeled by ^{125}I-calmodulin, as identified by their R_f on autoradiograms at several different exposure times. The labeled polypeptide indicated by the bottom arrow (M_r, 46,000) is not clearly visible in the reproduction but was clearly detectable on overexposed autoradiograms. The top and bottom portions of the gel and autoradiogram, which revealed no protein bands, were omitted from this figure. In other studies, calmodulin fragments of molecular weights as low as 8,000 have been detected by using this electrophoretic system. The prominent M_r 20,000 band is soybean trypsin inhibitor.

electrophoretic patterns of aliquots of the glycerol gradient fractions, sedimented with calmodulin in a 1:1:1 complex with a sedimentation coefficient of 5 S. Phosphoprotein phosphatase sedimented together with calcineurin, although a small amount of enzyme sedimenting more slowly than calcineurin was detected. This light form of the enzyme is believed to be associated with calcineurin that has been partially degraded during the purification and handling procedures. Its enzyme activity, like that of native calcineurin (Table 1), was inhibited by EGTA.

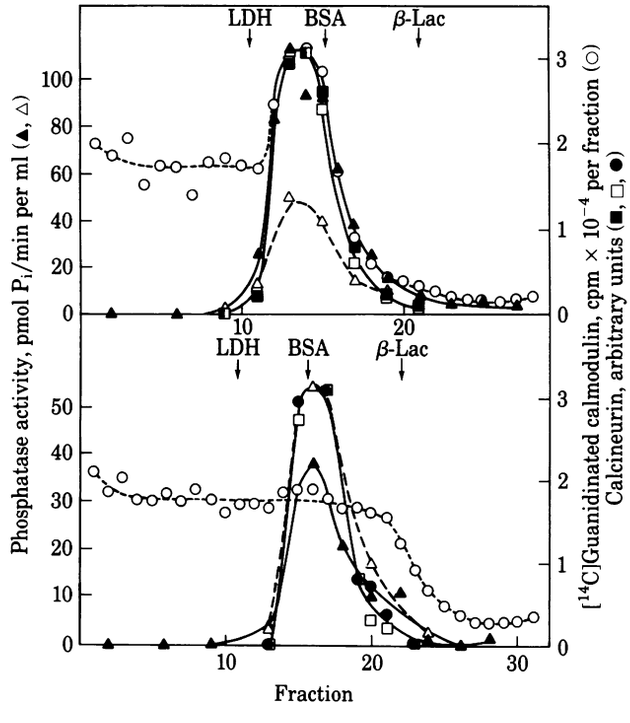

FIG. 3. Glycerol gradient centrifugation of calcineurin and trypsin-treated calcineurin in the presence of Ca^{2+} and [^{14}C]guanidinated calmodulin. Trypsin treatment was for 40 min at 30°C in the absence of calmodulin. (*Upper*) Calcineurin, (*Lower*) trypsin-treated calcineurin. ○, [^{14}C]Guanidinated calmodulin. Phosphatase activity was assayed in the presence of 0.2 mM EGTA (△) or 1 mM $CaCl_2$ and 1 μM calmodulin (▲). Calcineurin B (□), calcineurin A (■), and the M_r 45,000 fragments of calcineurin A (●) were quantitated by densitometric analysis. Sedimentation markers were lactate dehydrogenase (LDH), bovine serum albumin (BSA), and β-lactoglobulin (β-Lac).

Trypsin-treated calcineurin exhibited a high activity in the presence of EGTA and was inhibited by the addition of Ca^{2+} or Ca^{2+} and calmodulin (Table 1). When the trypsin-treated enzyme was subjected to glycerol gradient centrifugation in the presence of Ca^{2+} and calmodulin, no complex with labeled calmodulin was detected (Fig. 3 *Lower*). Protein phosphatase activity sedimented at 4.3 S with the complex of the two subunits of trypsin-treated calcineurin, the 45,000 M_r derivative of calcineurin A and calcineurin B. In this case, protein phosphatase activity was 2-fold greater in the presence of EGTA than in the presence of Ca^{2+} and calmodulin.

When glycerol gradient centrifugations were performed in the presence of EGTA and in the absence of calmodulin, protein phosphatase activity again sedimented with calcineurin or its proteolytic derivative (Fig. 4). Native calcineurin had a sedimentation coefficient of 4.5 S, slightly smaller than that ob-

Table 1. Effects of limited proteolysis on regulation of the phosphatase activity of calcineurin by Ca^{2+} and calmodulin

	Phosphatase activity, nmol P_i/min per mg	
Assay conditions*	Calcineurin	Trypsin-treated calcineurin†
EGTA (0.2 mM)	0.39 ± 0.08	3.19 ± 0.04‡
$CaCl_2$ (1 mM)	0.51 ± 0.04	2.47 ± 0.24
$CaCl_2$ (1 mM) + calmodulin (1 μM)	2.14 ± 0.06	1.76 ± 0.11

Results are presented as mean ± SEM.

* Phosphatase activity was found to vary slightly (up to 2-fold) with different preparations of [^{32}P]phosphorylated myosin light chains. Soybean trypsin inhibitor has no effect on enzyme activity in the presence or absence of calmodulin.

† Trypsin-treated enzyme was obtained after 40-min treatment with trypsin in the absence of calmodulin.

‡ After freezing and thawing once, the activity of the enzyme was reduced by 40–60% and was no longer affected by EGTA, Ca^{2+}, or Ca^{2+}·calmodulin.

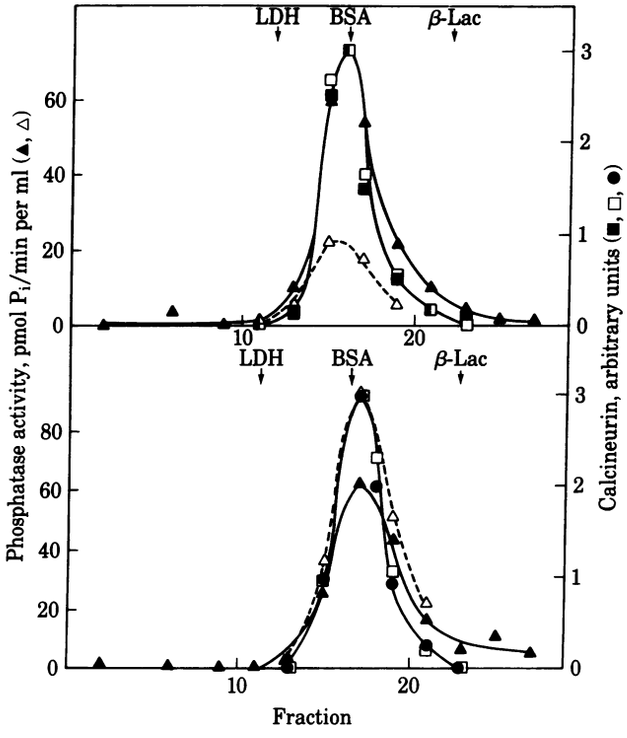

FIG. 4. Glycerol gradient centrifugation of calcineurin in the presence of EGTA. Calcineurin (*Upper*) and trypsin-treated calcineurin (*Lower*) were analyzed by glycerol gradient centrifugation as described in the legend to Fig. 3. The gradient solutions contained 1 mM EGTA in the absence of added Ca^{2+} or calmodulin. Phosphatase activity was assayed in the presence of EGTA (△) or 1 mM $CaCl_2$ and 1 μM calmodulin (▲). Calcineurin B (□), calcineurin A (■), and the M_r 45,000 fragment of calcineurin A (●) were quantitated by densitometric analysis.

served for the calcineurin–calmodulin complex (Fig. 3 *Upper*). In contrast, the trypsin-treated enzyme exhibited the same sedimentation coefficient (4.3 S) in the presence of EGTA as that measured in the presence of Ca^{2+}·calmodulin. The trypsin-treated enzyme sedimented more slowly than the intact enzyme (Fig. 4 *Lower*).

DISCUSSION

The present results provide further evidence supporting the identification of calcineurin as a protein phosphatase: (*i*) a Ca^{2+}- and calmodulin-stimulated phosphatase activity sediments at 4.5 S with calcineurin in glycerol gradients; (*ii*) in the presence of Ca^{2+}·calmodulin, phosphatase activity sediments at 5.0 S with the calmodulin–calcineurin complex; (*iii*) after limited proteolysis, phosphatase activity sediments at 4.3 S with trypsin-treated calcineurin; (*iv*) loss of calmodulin binding to calcineurin resulting from proteolysis is accompanied by loss of calmodulin stimulation of phosphatase activity; and (*v*) like that of calcineurin, the rate of tryptic digestion of the protein phosphatase is decreased by the presence of calmodulin.

Limited proteolysis of calcineurin with trypsin specifically alters the large subunit, calcineurin A. Tryptic digestion of calcineurin in the absence of calmodulin reveals a protease-sensitive domain on calcineurin A that is cleaved rapidly, removing approximately one-fourth of the large subunit. This entire domain is apparently highly susceptible to proteolysis, because no peptide corresponding to the cleaved domain can be detected after only 4 min of digestion. Loss of this domain is accompanied by loss of ability of the subunit to bind calmodulin. With long periods of digestion, additional cleavages, which may be occurring at either end of the fragment, result in generation of slightly smaller species. These fragments of calcineurin A remain in association with calcineurin B in a 1:1 complex, in the presence or absence of Ca^{2+}.

Inclusion of calmodulin in the digest mixture renders calcineurin more resistant to proteolysis. Even in the presence of a 3-fold greater concentration of trypsin, the highly susceptible domain on calcineurin A is protected from proteolysis. Instead, cleavages near the NH_2 or COOH termini generate several large fragments of calcineurin A that retain the ability to bind calmodulin. After long incubation, calcineurin A is further digested to fragments of M_r 46,000 and 40,000. The M_r 46,000 fragment retains the ability to bind calmodulin, suggesting that this polypeptide is generated as a result of cleavage at the terminus opposite that of the calmodulin-binding domain. A loss of calmodulin regulation of the phosphatase is observed only after long incubation times, when 50% of the calmodulin binding activity measured by the gel overlay method is lost. However, the contribution of multiple proteolytic species, potentially subject to differing regulation, precludes simple correlation between loss of calmodulin binding and loss of stimulation. In contrast, when the enzyme is treated with trypsin in the absence of calmodulin, rapid loss of calmodulin binding and stimulation is observed after only 4 min of digestion. The trypsin-treated enzyme has not lost its ability to be regulated by Ca^{2+}, because its high level of activity can still be inhibited by Ca^{2+} (Table 1). Although the mechanism of this Ca^{2+} inhibition is not yet understood, it is consistent with the observation that the enzyme contains an intact Ca^{2+}-binding subunit, calcineurin B. The observation that trypsin-treated calcineurin undergoes aggregation in the presence of Ca^{2+} (unpublished observation) could also explain the inhibition of enzyme activity by Ca^{2+}.

The phosphatase activity of trypsin-treated calcineurin is inhibited further by calmodulin, suggesting that the freshly digested enzyme is still able to interact with calmodulin. The inability to demonstrate significant interaction of trypsin-treated calcineurin with calmodulin does not exclude the possibility of a low-affinity interaction, which could escape detection under the conditions of gel overlay and gradient sedimentation in the presence of glycerol. In view of the homology between calmodulin and myosin light chains, it is conceivable that the inhibition of phosphatase activity results from calmodulin acting as a competitive inhibitor at the catalytic site, which may be accessible to calmodulin only after the calmodulin-binding domain is removed by limited proteolysis. Indeed, after limited proteolysis, myosin light chain kinase, which also uses myosin light chains as a substrate, exhibits similar inhibition by calmodulin (21). In contrast, trypsin-treated phosphodiesterase is not inhibited by calmodulin (10). In the present case, the inhibitory effect of calmodulin on trypsin-treated calcineurin is lost upon storage, making a more detailed analysis of this phenomenon difficult.

The results of limited proteolysis support the identification of at least two domains within calcineurin A. One domain contains the catalytic site as well as the site for interaction with calcineurin B. This region of calcineurin A is resistant to trypsin, possibly resulting from protection afforded by the stable association with calcineurin B. Thus, further resolution of this polypeptide into potentially distinct catalytic and calcineurin B-binding domains was not observed. The other domain, which is highly susceptible to proteolysis, appears to be essential for interaction with calmodulin. By analogy with other calmodulin-binding proteins (10, 21–28), present results are consistent with the presence of a calmodulin-binding domain, whose inhibitory effect on enzyme activity is removed either by proteolysis or as

a result of conformational changes attendant to calmodulin binding.

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