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MODULATION OF CALCINEURIN PHOSPHOTYROSYL PROTEIN PHOSPHATASE ACTIVITY BY CALMODULIN AND PROTEASE TREATMENT

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SUMMARY: Calcineurin (CN) dephosphorylated [^{32}P] phosphotyrosyl glutamine synthetase, a model phosphoprotein substrate containing ~ 1 mol of phosphotyrosine per mol subunit. Phosphatase activity with and without calmodulin (CaM) was greatly stimulated by Mn^{2+}; with Ca^{2+}, even in the presence of CaM, activity was very low. CaM-stimulated phosphatase activity exhibited deactivation with time; initial rates declined markedly after 2-3 min. The Michaelis constant for substrate (3 μM) was identical whether 2 or 12 min assays (with CaM) were used suggesting that the decreased rate of hydrolysis did not result from a decrease in affinity for the phosphoprotein substrate. Limited proteolysis of CN by chymotrypsin increased phosphatase activity 2-3 times that of CaM-supported activity; however, addition of CaM to assays with protease-activated CN reduced activity to that observed for non-proteolyzed enzyme. These data suggest that, in addition to stimulation, CaM can inhibit certain activated conformations of the phosphatase.

Calcineurin (CN) , a Ca^{2+}- and calmodulin (CaM)-binding protein (1), exhibits phosphatase activity toward several phosphoproteins (2-11) as well as p-nitrophenyl phosphate (12). Activation by divalent cations (Ca^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}), with and without CaM, has been reported (1-6, 13-16). Recent reports showed that CN dephosphorylated phosphotyrosine and peptides or proteins containing phosphorylated tyrosine residues (12-15). To investigate the phosphatase activity of CN toward phosphotyrosyl protein, we have used a unique substrate, [^{32}P] phosphotyrosyl glutamine synthetase, prepared by enzymatic removal of the nucleoside moiety from the denatured adenylylated protein (17). This substrate,

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Abbreviations used in this paper: DEAE, diethylaminoethyl; EGTA, ethyl glycol bis (β-aminoethyl ether) N, N, N', N' tetraacetic acid; CaM, calmodulin; CN, calcineurin.

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which is a well-characterized protein with one phosphotyrosine per subunit, is capable of being dephosphorylated by cellular phosphotyrosyl protein phosphatases (18). Some kinetic and regulatory properties of the native and chymotrypsin-treated CN phosphatase with this substrate are described, which suggest that it may serve as a model phosphotyrosyl protein substrate.

Materials and Methods

Purification of calcineurin and calmodulin. CN was purified to homogeneity from bovine brain as described (19). The procedure, involving ion-exchange chromatography at pH 5.5 on DEAE Bio-Gel A (Bio-Rad), chromatography on Cibacron-blue-substituted agarose (Affi-Gel Blue, Bio-Rad), and affinity chromatography with CaM-Sepharose, is similar to that of Sharma et al. (20); however, only stepwise elutions were used. The purified protein behaved as a single hydrodynamic species in the ultracentrifuge (M_r ~ 78 kDa) and on gel filtration (Stokes radius ~ 37 nm). Under denaturing conditions, electrophoresis separated two peptides of 60 and ~ 18 kDa in approximately equal proportions, which accounted for > 95% of the Coomassie blue staining material. Homogenous CaM, prepared as described (21), had ultraviolet and circular dichroic spectra identical to those published.

Preparation of [^{32}P] phosphotyrosyl glutamine synthetase. [^{32}P] phosphotyrosyl glutamine synthetase, prepared by micrococcal nuclease treatment of denatured, carboxymethylated [^{32}P] adenylylated glutamine synthetase (17), contained ~ 1 mol of phosphate/mol of 50 kDA subunit (~ 40 Ci/mol). Since in the preparation of this derivative the protein was completely denatured in urea and treated with iodoacetic acid to block free sulfhydryl groups, it is incapable of forming oligomers and exists exclusively as a monomeric species. The specific activity of the phosphoprotein was confirmed by analytic determination of tyrosine phosphate following base hydrolysis (22). It was stored frozen after dialysis against 1 mM sodium borate, pH 9.3. Spontaneous hydrolysis of the phosphotyrosyl bond was negligible for at least one month.

Assay of phosphatase activity. Assays (total volume 50 µl) contained 50 mM Tris HCl (pH 8.0), 0.1 mM EGTA, 1.0 mM $MnCl_2$, 1.0 mM $CaCl_2$, 6 µM CaM, 3 µM [^{32}P] phosphotyrosyl glutamine synthetase, and bovine serum albumin, 1 mg/ml, unless otherwise noted. Reactions were initiated by addition of enzyme, incubated at 30°C, and terminated by addition of 550 µl of 20% (w/v) trichloroacetic acid. After two min, samples were centrifuged in a Beckman Microfuge (2 min) and 550 µl of the supernatant was removed for radioassay. Blanks containing all components except enzyme were < 1% of the total ^{32}P added.

Materials. Ultrapure Tris base was purchased from BRL, soybean trypsin inhibitor and chymotrypsin from Worthington, bovine serum albumin (Pentex) from Miles, and trichloroacetic acid from Baker. $CaCl_2$ and $MnCl_2$ were Puratronic grade from Curtis Matthey Lt. EGTA (Eastman Kodak) was recrystallized from water.

Results

Hydrolysis of 3 µM [^{32}P] phosphotyrosyl glutamine synthetase was proportional to CN concentration up to a maximum of 25% substrate utiliza-

Table I

Effect of Ca^{2+}, Mn^{2+}, and CaM on Phosphatase Activity

Additions	Phosphatase Activity (pmol/min)
$CaCl_2$	0.037
$CaCl_2$, CaM	0.188
$MnCl_2$	1.38
$MnCl_2$, CaM	3.49
$CaCl_2$, $MnCl_2$, CaM	4.10

CN (0.6 μM) was assayed for 10 min as described in Materials and Methods except that 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 6 μM CaM were present as indicated.

tion (data provided). In the absence of added divalent cations, hydrolysis was negligible and with 1 mM $CaCl_2$ it was very low (Table I). Addition of CaM in the presence of Ca^{2+} increased activity five-fold. With Mn^{2+} alone, activity was > 20 times that with Ca^{2+} alone and the addition of CaM stimulated activity about three-fold (Table I). When assayed with 5 mM $MgCl_2$ instead of Mn^{2+}, maximal activity was reduced ~ 4-fold although the apparent CaM stimulation was greater (~ 10 fold); this CaM-dependent activity was inhibited by 100 μM trifluoperazine or W-7 (data not shown). Although protein phosphatase activity was proportional to enzyme concentration, it was not constant with time despite the fact that less than 25% of substrate was hydrolyzed (Fig. 1). At several enzyme concentrations, the initial rate of hydrolysis decreased markedly after 2-3 min (Figs. 1 and 2) and then remained constant at a rate equal to or greater than that observed in the absence of CaM (Fig. 2)[1]. In assays without CaM, activity was constant throughout the assay period (Fig. 2, inset). The non-linear time course was not altered by incubation of the phosphatase for 5 min at 30°C in 50 mM Tris-HCl, pH 8.0, before assay.

[1] Based on specific activities, the final CaM-supported rate in Fig. 2 was ~ 50-60% greater than that in the absence of CaM.

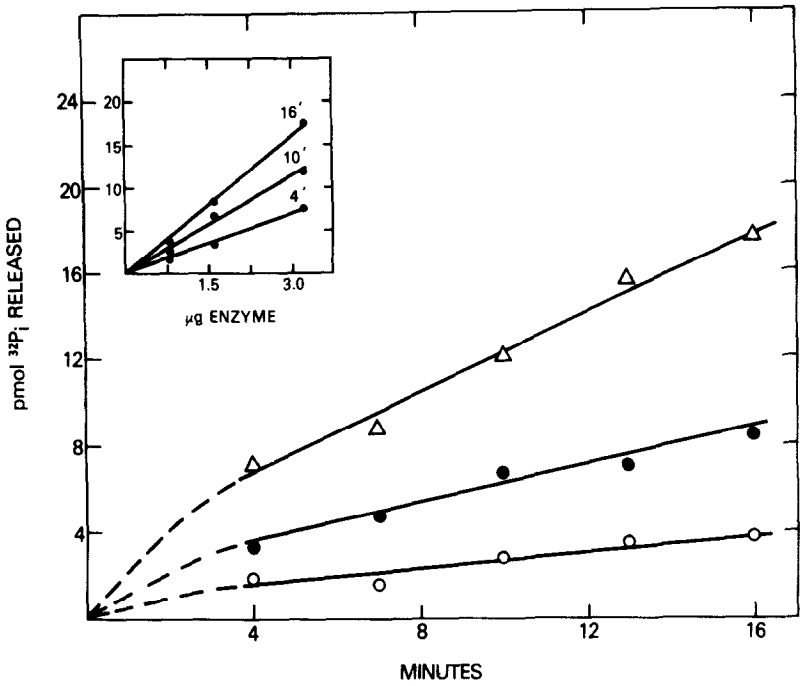

Fig. 1. Time course of $^{32}P_i$ release at three phosphatase concentrations. CaM-stimulated phosphatase activity of CN was assayed for the indicated times as described in Materials and Methods except that substrate and CaM concentrations were 6 and 2 μM, respectively. Activity was assayed with 0.8 μg (○), 1.6 μg (●), and 3.2 μg (△) CN. Inset: $^{32}P_i$ released at 4, 10, and 16 min as a function of amount of CN.

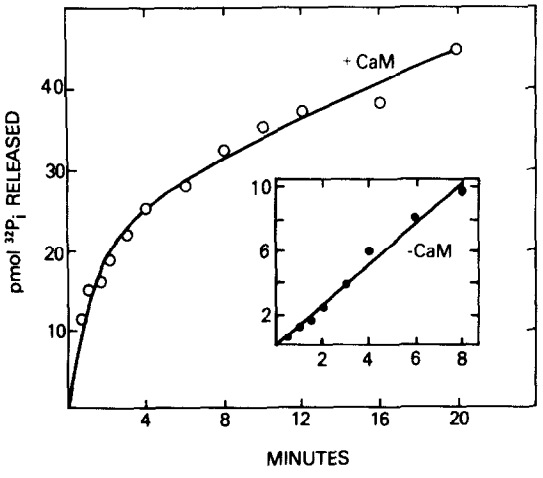

Fig. 2. Time course of $^{32}P_i$ release with and without CaM. CaM-stimulated phosphatase activity (○) of CN (0.6 μM) was assayed for the indicated times as described in Materials and Methods. Inset: Activity in assays without CaM (●), with 6 μM substrate and 1 μM CN.

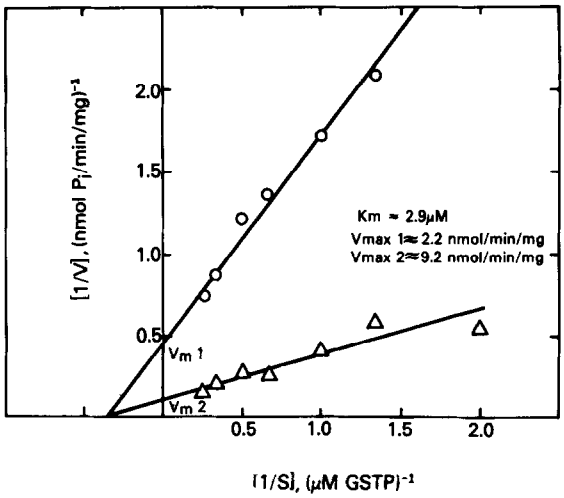

Fig. 3. Effect of substrate concentration on phosphatase activity. CaM-stimulated phosphatase activity of CN (0.7 μM) activity was assayed for 2 (△) or 12 min (○) as described in Materials and Methods except that the substrate concentration was varied (0.5-4 μM) as indicated.

The dependence of phosphatase activity on substrate concentration was comparable whether the enzyme was assayed for 2 or 12 minutes (Fig. 3), suggesting that the change in activity with time was not a result of drastic changes in its affinity for substrate. Lineweaver-Burk plots were linear with an apparent K_m of ~ 3 ± 1.2 μM in four experiments with two different preparations of substrate and three different enzyme preparations. The apparent V_{max} depended on the length of the assay (Fig. 3) with a maximal rate of 9-10 nmol/min/mg enzyme when assayed for 2 min and about 1/4 that when assayed for 12 minutes.

When CN was treated with increasing concentrations of α-chymotrypsin, phosphatase activity increased dramatically corresponding to the production of several smaller peptides of the catalytic A subunit (Fig. 4). The activity of the protease-treated sample, when assayed with Mn^{2+} and Ca^{2+} was 2-3 times that of untreated sample assayed with Mn^{2+}, Ca^{2+}, and CaM; addition of CaM to such highly activated samples reduced activity to a level comparable to that of control enzyme assayed with CaM. The increase in CaM-supported activity observed for sample treated with the highest concentration of chymotrypsin was correlated with the appearance of a

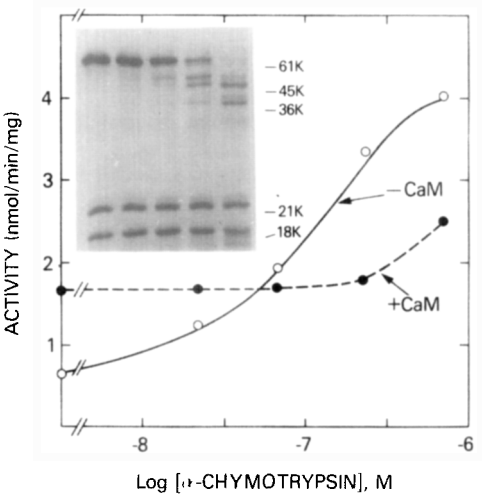

Fig. 4. Effect of chymotrypsin on phosphatase activity of CN assayed in the presence and absence of CaM. Samples of CN (18 µg, ~ 4.5 µM final concentration) were incubated with the indicated concentrations of α-chymotrypsin in a total volume of 55 µl containing 20 mM Tris, pH 8.0, 0.1 mM EGTA, 1 mM $MgCl_2$, and 10% glycerol. After 3 min at 30°C, proteolysis was terminated by addition of 20 µl of 1 mg/ml soybean trypsin inhibitor. Phosphatase activity was assayed for 4 min as described in Materials and Methods in the absence (○) or presence (●) of 6 µM calmodulin; reactions were initiated with 15 µl of the protease-treated sample. An equal portion of each sample was precipitated with trichloroacetic acid (10% v/v) for SDS gel electrophoresis shown in the inset. Note: The peptide with M_r ~ 21 kDa is the added soybean trypsin inhibitor.

36 kDA peptide, suggesting that this fragment may no longer be subject to CaM-dependent inhibition.

Discussion

The CaM-activated phosphatase activity of CN, first reported by Stewart et al. (2) has been demonstrated with several phosphoprotein substrates (2-11); however, the physiological substrate(s) for the CaM-stimulated phosphatase has not been identified. With growing information on tyrosine kinases and the role of phosphotyrosyl proteins, it was of interest to study a defined model substrate. [^{32}P] phosphotyrosyl glutamine synthetase, initially described by Martensen and Stadtman (17), appeared to be especially suited for this purpose since it is a well-characterized, monomeric protein (M_r = 50,000) with a known amino acid sequence around the phosphotyrosine site, -Met-Asp-Lys-Asn-Leu-Tyr(P)-Asp-Leu-Pro-Pro-Gln- (23).

The activity of CN toward this substrate is consistent with general regulatory properties of the phosphatase. Activity is greatly stimulated by Mn^{2+}, whereas Ca^{2+} supports only modest enzyme activity even in the presence of CaM. Since this apparently has not been observed by some groups (2-4, 6), it may be that the relative effectiveness of divalent cations is, at least in part, dependent on substrate or other assay conditions; this consideration was also suggested by King and Huang (6).

A marked decline, within 2 to 3 min, of the initial rate of catalysis was observed in assays with, but not without, CaM. Since basal rates were constant, this reflects a rapid reduction in the degree of CaM stimulation. Indeed, the apparent rate in the presence of CaM was usually 10-15 times that in its absence when measured for one minute, but only three times when measured after ten minutes. This apparent "deactivation" agrees with the findings of King and Huang (16), using p-nitrophenyl phosphate as substrate. Prior incubation of CN did not alter this behavior, suggesting that it is not the result of a simple inactivation process. The finding that the apparent Michaelis constants were the same whether assays for 2 or 12 min were used makes it unlikely that the decrease in rate of reaction results from a change in substrate affinity. All of our observations suggest that is the same CaM-dependent regulatory property of the enzyme described using the chromogenic substrate (16).

In our studies, chymotrypsin activated phosphoprotein phosphatase activity well beyond that observed with CaM suggesting latent enzyme activity not expressed without proteolysis. Manalan and Klee (4), using trypsin-treated CN, also observed a higher activity after protease-treatment when compared to control CaM-supported activity although Tallant and Cheung (24) did not observe such an effect. Based upon analysis of the peptides observed on SDS gel electrophoresis, it would appear that degradation of the catalytic 61 kDa A subunit to fragments of 48 and/or 45 kDa greatly increased activity. These peptides were evidently capable of still interacting with CaM, which reduced activity to the same level

as for control CaM-supported activity; this suggests an interaction with a domain on the phosphatase which attenuates the proteolytically-activated state. With subsequent production of a 36 kDa peptide, activity assayed with CaM increased somewhat, consistent with an inability of this species to interact with CaM.

The apparent K_m for this phosphotyrosyl substrate was ~ 3 μM, in rough agreement with a number of non-tyrosyl phosphoprotein substrates (8, 9, 24). The value of ~ 10 nmol/min/mg for the Mn^{2+}-supported V_{max} is comparable to those for phosphohistone H_1 and myosin light chains (4, 6) when assayed at micromolar concentrations, and with those recently reported for several phosphotyrosyl protein substrates (25). Since both the regulatory and kinetic properties of CN using phosphotyrosyl glutamine synthetase are similar to those with other phosphoproteins, this phosphotyrosyl protein may prove useful as a model substrate for this enzyme.

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