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Stoichiometric methylation of calcineurin by protein carboxyl *O*-methyltransferase and its effects on calmodulin-stimulated phosphatase activity

(post-translational modification/calmodulin-binding proteins/carboxyl methylation)

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ABSTRACT **Calcineurin, a calmodulin-stimulated protein phosphatase, was a substrate for purified bovine brain protein carboxyl *O*-methyltransferase (protein *O*-methyltransferase; EC 2.1.1.24) and incorporated up to 2 mol of CH_3 per mol of calcineurin. Carboxyl methylation was dependent on the concentrations of *S*-adenosyl-L-[*methyl*-^{3}H]methionine and was prevented by addition of the carboxyl methylation inhibitor *S*-adenosylhomocysteine. The stoichiometry of methyl group incorporation was related to the ratio of methyltransferase/calcineurin. The rate of spontaneous hydrolysis of carboxyl methylester groups on calcineurin increased rapidly above pH 6.5 with those on native carboxyl-methylated calcineurin substantially more labile than for tricholoracetic acid-precipitated calcineurin. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (pH 2.4) confirmed that the A subunit of calcineurin (M_r = 61,000) was the primary site of carboxyl methylation with little, if any, modification of the B subunit (M_r = 18,000). When carboxyl-methylated calcineurin (≈1–2 mol of CH_3 per mol of protein) was assayed for *p*-nitrophenyl phosphatase activity at pH 6.5, a marked inhibition of calmodulin-stimulated activity was observed while there was little effect on Mn^{2+}-stimulated phosphatase activity. Thus, calcineurin appears to be an excellent substrate for protein carboxyl *O*-methylation and this modification, which impairs calmodulin stimulation of phosphatase activity, may be of functional significance.**

Carboxyl methylation of glutamic and/or aspartic acid residues of proteins by the enzyme protein carboxyl *O*-methyltransferase (protein *O*-methyltransferase; EC 2.1.1.24) is well documented (1–3), although the functional significance of this reversible modification of proteins remains an enigma (4). It has been suggested that carboxyl methylation can influence neurotransmitter synthesis and release (5–7), leukocyte chemotaxis (8, 9), and repair/recognition of D-aspartate residues in protein (10–15). More recently, effects of carboxyl methylation on the function of calmodulin (16–18) and calmodulin-binding proteins have been investigated (19, 20). Carboxyl methylation of calmodulin was observed both *in vivo* (16, 18) and *in vitro* (16–20). However, carboxyl methylation of calmodulin was consistently substoichiometric (18, 20, 21), as is the case for other substrates (10). We have observed that proteins eluted from a calmodulin-Sepharose affinity column are a good source of methyl acceptor proteins (20). Purified bovine brain Ca^{2+}/calmodulin-stimulated phosphodiesterase served as a substrate for protein carboxyl *O*-methyltransferase, and calmodulin-stimulated, but not basal, activity was attenuated following carboxyl methylation (20). Since calcineurin (22), a calmodulin-activated phosphatase (23, 24), is the major calmodulin-binding protein in brain, it was of interest to examine whether this protein can serve as a substrate for protein carboxyl *O*-methyltransferase. As reported here, calcineurin can be carboxyl methylated to an extent of 2 mol/mol of protein with resulting inhibition of calmodulin-stimulated phosphatase activity.

METHODS

Materials. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (Ado[^{3}H]-Met) (62 Ci/mmol and 15 Ci/mmol; Ci = 37 GBq) was purchased from Amersham; 2-(*N*-morpholino)ethanesulfonic acid (Mes) was from Calbiochem; AdoMet and *S*-adenosylhomocysteine (AdoHcy) were from Sigma; AdoHcy-agarose was from Bethesda Research Laboratories; electrophoresis supplies were from Bio-Rad; and frozen bovine brains were from Hazelton Labs (Denver, PA). All reagents were of the highest available purity. Calmodulin-Sepharose was prepared according to the method of Kincaid and Vaughan (25).

Enzyme Preparations. Bovine brain protein carboxyl *O*-methyltransferase was purified to apparent homogeneity by using minor modifications of previously published techniques (26, 27). Bovine brain calcineurin was purified to homogeneity (28) and contained subunits of $M_r \approx 61{,}000$ and $M_r \approx 18{,}000$ in approximately equal proportions, which accounted for ≈95% of Coomassie-stained protein after $NaDodSO_4$ gel electrophoresis.

Carboxyl Methylation Assays. Reactions were carried out in a final volume of 100 μl in 1.5-ml microcentrifuge tubes and contained the following: calcineurin (1.2–62.5 pmol), protein carboxyl *O*-methyltransferase (2–13.5 μM), and Ado[^{3}H]Met (0.1–200 μM) in 50 mM Mes (pH 6.25). The reaction rate was constant for 15 min at 37°C, and this time was used for most assays. When carboxyl methylation of calcineurin was examined as a function of Ado[^{3}H]Met concentration, the specific activity was constant (33,300 dpm/pmol; using 15 Ci/mmol of Ado[^{3}H]Met).

Reactions were terminated by addition of 1.0 ml of ice-cold 10% trichloroacetic acid and 100 μg of bovine serum albumin. After 5 min at 4°C, the samples were centrifuged for 2.5 min in a microcentrifuge (10,000 × *g*), and the supernatant was aspirated. Carboxyl methylesters were hydrolyzed by incubating the pellets in 500 μl of 1.0 M sodium borate (pH 11) for 20 min at 37°C, and the resulting [^{3}H]methanol was extracted into 3 ml of isoamyl alcohol/toluene, 2:3 (vol/vol), with 5% methanol and radioactivity was counted. Nonvolatile radioactivity was <10% of the total and was not usually determined.

In studies designed to determine stoichiometry, the trichloroacetic acid-precipitated pellet was resuspended and

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Abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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centrifuged three times with 10% trichloroacetic acid and then with acetone. This pellet was then solubilized in 1.5 M Tris·HCl, pH 8.8/0.4% $NaDodSO_4$ for radioassay.

Determination of Carboxyl Methylester Stability. Carboxyl methylation was achieved by incubation of 1.0 μg (12.5 pmol) of calcineurin with 4.0 μM bovine protein carboxyl *O*-methyltransferase and 5 μM Ado[^{3}H]Met in 50 mM Mes (pH 6.25) for 15 min at 37°C. In one experiment, the reaction was terminated by addition of 1.0 ml of 10% trichloroacetic acid and 50 μg of bovine serum albumin. After centrifugation, the pellet was washed with 1.0 ml of 10% trichloroacetic acid and with acetone. This pellet was then resuspended in 200 μl of either 150 mM Hepes (pH 7.5), 150 mM Hepes (pH 7.0), or 150 mM Mes (pH 6.25) and incubated at 37°C; samples were removed at specified times for determination of trichloroacetic acid-precipitable radioactivity.

In a second experiment, calcineurin (1.0 μg) was carboxyl methylated in the same way but the reactions were stopped by addition of 20 μl of 1 mM AdoHcy. As before, the following final concentrations of buffer were added to give a total volume of 200 μl: 150 mM Hepes (pH 7.5), 150 mM Hepes (pH 7.0), and 150 mM Mes (pH 6.25). The reaction mixtures were then incubated at 37°C for specified times and aliquots were removed for determination of trichloroacetic acid-precipitable radioactivity.

Assay of Calcineurin Phosphatase Activity. The *p*-nitrophenyl phosphatase activity of calcineurin (29) was measured at pH 6.5 in order to minimize hydrolysis of preformed methylesters. Carboxyl methylation reactions contained 0.5 μg (6.25 pmol) of calcineurin, 0.1 mM EGTA, 200 μM AdoMet, and 50 mM Mes buffer (pH 6.5) in a final volume of 50 μl with or without purified bovine brain protein carboxyl *O*-methyltransferase (9–14 μM). After 15 min at 30°C, 200 μM AdoHcy was added, and the reaction tubes were frozen on dry ice‡ before assay of phosphatase activity. Prior to assay of phosphatase activity, 1 mM Mn^{2+}, 1 mM Ca^{2+}, and 100 mM *p*-nitrophenyl phosphate in 50 mM Mes buffer (pH 6.5) (final concentrations) were added to the frozen reaction mixture in a volume of 10 μl; for assays of calmodulin-stimulated activity, calmodulin (2 μM) was present in the addition. The samples were rapidly thawed, mixed by a 1.0-min centrifugation (10,000 × *g*), and then incubated at 30°C for 3 min. Reactions were terminated by addition of 300 μl of 2 M Na_2CO_3 (final volume = 360 μl). Release of product was determined by measuring absorbance at 410 nm on a Beckmann DU-8B spectrophotometer, and phosphatase activity (μmol of product per mg of calcineurin × min^{-1}) was determined by using the molar extinction coefficient for *p*-nitrophenol (E^M_{410} = 1.82 × 10^4). Values were also expressed as percent stimulation of phosphatase activity in the presence of calmodulin.

Parallel samples of calcineurin were used to determine the stoichiometry of methyl transfer, using conditions identical to those employed in the measurement of calcineurin phosphatase activity, with the exception that Ado[^{3}H]Met (200 μM; 1100 dpm/pmol) was included in the reaction mixture. The reactions were terminated by addition of 1.0 ml of 10% tricholoracetic acid and acid-precipitable radioactivity was determined.

Protein content was measured by using the method of Bradford (30).

Acidic Gel Electrophoresis. Since carboxyl methylesters are unstable at neutral and basic pH, an acidic $NaDodSO_4$ gel electrophoresis system was employed to investigate carboxyl methylation of calcineurin subunits as described (14, 31).

‡Calcineurin phosphatase activity of nonmethylated samples was not affected by one freeze-thaw cycle, although repeated freeze-thawing resulted in a loss of activity; thus, calcineurin that had been frozen only after original portioning was employed for each experiment.

RESULTS

Carboxyl Methylation of Calcineurin. When calcineurin (1.2–62.5 pmol) was incubated for 15 min at 37°C with 5 μM Ado[^{3}H]Met and 4.0 μM purified protein carboxyl *O*-methyltransferase, a concentration-related increase in the formation of methylesters was noted (Fig. 1). When the concentration of Ado[^{3}H]Met was varied at fixed concentrations of calcineurin (12.5 pmol) and protein carboxyl *O*-methyltransferase (4.0 μM), methylester formation was dependent on AdoMet concentration, reaching saturation at 5 μM (Fig. 1 *Inset*). The K_m for AdoMet calculated from double reciprocal plots was 0.5 μM (data not shown), with a V_{max} of 253 pmol of CH_3 transferred per mg of enzyme × min^{-1}; the K_m value for AdoMet is comparable to those determined by using other protein substrates (1).

Up to 2 mol of CH_3 could be incorporated per mol of calcineurin with the extent of incorporation directly related to the ratio of methyltransferase/calcineurin (Fig. 2). When the ratio of methyltransferase/calcineurin was >100:1, stoichiometry of 1 mol of CH_3 per mol of calcineurin or greater was achieved. At lower ratios of methyltransferase/calcineurin, a stoichiometry of 0.3–0.4 mol of CH_3 per mol of calcineurin was achieved.

Stability of Carboxyl Methylesters on Calcineurin. When calcineurin was carboxyl methylated and the reaction was stopped with excess AdoHcy, there was a dramatic pH-dependent loss of carboxyl methylesters, with an apparent half-life of 30.0 min at pH 6.5, decreasing to 3.0 min at pH 7.5 (Fig. 3). This loss of methylesters at pH 7.5 was complex, with a rapid loss of methylesters during the first 5 min of incubation, after which the decay was quite slow; a similar trend was also observed at pH 6.25 and pH 7.0. When carboxyl-methylated calcineurin was acid-precipitated following the enzymatic reaction, the spontaneous hydrolysis of methylesters, especially at pH 7.0 and pH 7.5, was much slower. This suggests that the faster rate of decay observed at those pHs was related to a labile methylester environment found in the native tertiary structure of the protein.

After freezing at −80°C for 24 hr, samples carboxyl methylated with a molar ratio of 66:1 (methyltransferase/

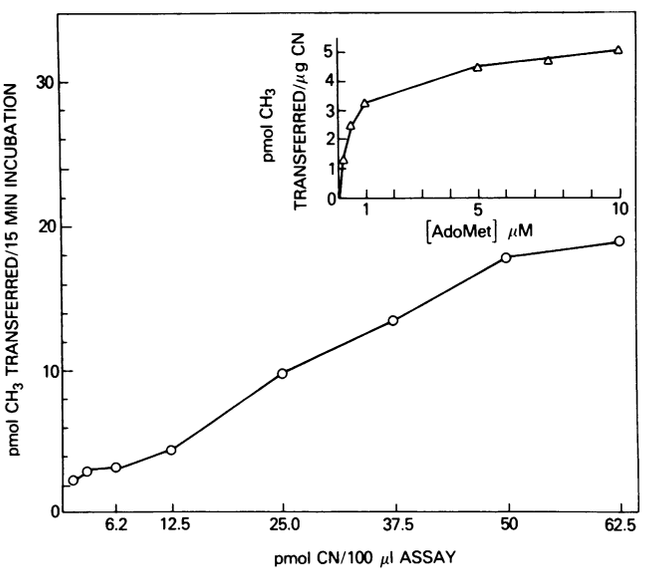

FIG. 1. Carboxyl methylation of calcineurin (CN). Purified bovine brain calcineurin was carboxyl methylated with 5 μM Ado[^{3}H]Met and 4.0 μM protein carboxyl *O*-methyltransferase for 15 min at 37°C. (*Inset*) The concentration of Ado[^{3}H]Met was varied, and calcineurin (12.5 pmol) was carboxyl methylated with 4.0 μM of bovine brain protein carboxyl *O*-methyltransferase. Each point represents the mean of duplicate values of a representative experiment.

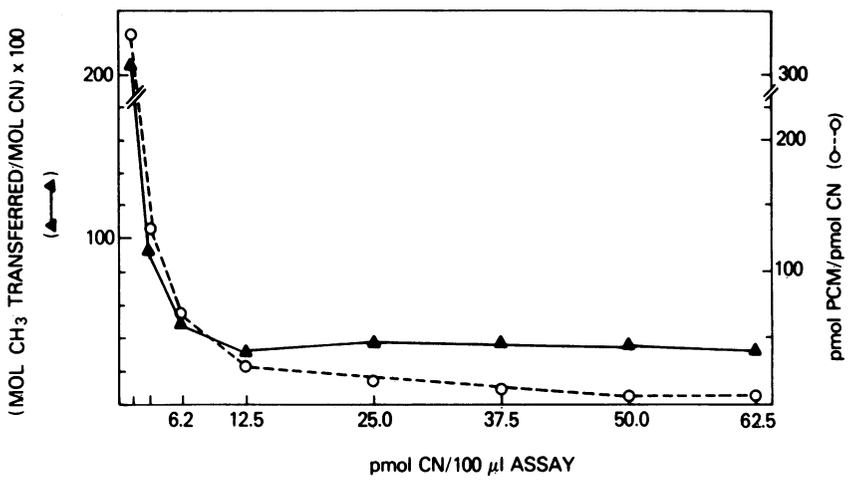

FIG. 2. Stoichiometry of methyl transfer to calcineurin (CN). Conditions were as described in the legend to Fig. 1. Protein carboxyl *O*-methyltransferase (PCM) was held constant at 4.0 μM, while the concentration of calcineurin was varied. The open circles show the ratio of protein carboxyl *O*-methyltransferase to calcineurin. Each point was determined in duplicate.

calcineurin) had 0.88 mol of CH_3 transferred per mol of protein, whereas samples with a ratio of 132:1 (methyltransferase/calcineurin) had 1.88 mol of CH_3 transferred per mol of protein; these values are comparable to those of samples that were not frozen. Thus, carboxyl-methylated calcineurin appeared stable to freezing, *per se*, and was frozen prior to assay of phosphatase activity.

Carboxyl Methylation of Calcineurin Subunits. To determine the subunit(s) of calcineurin that was carboxyl methylated, 4.0 μg of calcineurin was incubated with 4.0 μg of bovine brain protein carboxyl *O*-methyltransferase and 5.0 μCi of Ado[^{3}H]Met (62 Ci/mmol) in 50 mM Mes (pH 6.25). Samples were then electrophoresed under acidic (pH 2.4) conditions of NaDodSO$_4$/PAGE to ensure stability of the methylesters. The Coomassie blue staining pattern revealed three proteins corresponding to calcineurin A and B and protein carboxyl *O*-methyltransferase. As shown in Fig. 4, the A subunit of calcineurin (M_r = 61,000) was extensively carboxyl methylated, whereas the B subunit (M_r = 18,000) was methylated to a negligible extent; this reaction was inhibited by 100 μM AdoHcy (lane 3).§

Effects of Carboxyl Methylation on Calmodulin-Stimulated Phosphatase Activity of Calcineurin. Samples of calcineurin were incubated in the presence or absence of protein carboxyl *O*-methyltransferase for 15 min at 30°C, after which the reaction was stopped with excess AdoHcy and frozen on dry ice. Phosphatase activity was determined at pH 6.5 immediately after thawing the samples in order to minimize hydrolysis of preformed methylesters. Table 1 presents three experiments that demonstrate the marked inhibitory effect of carboxyl methylation on calmodulin-stimulated phosphatase activity. By contrast, methylation did not greatly diminish the Mn^{2+}-stimulated phosphatase activity;¶ indeed, in four experiments, methylation slightly increased this activity on the average (26.5% ± 19.6%). Thus, under such conditions in which at least 1:1 stoichiometry of carboxyl methylation was observed, a selective attenuation of calmodulin-stimulated phosphatase activity was noted, suggesting modification of its regulatory properties.

DISCUSSION

Calcineurin, a calmodulin-stimulated phosphatase, was enzymatically carboxyl methylated with incorporation of up to 2 mol of CH_3 per mol of protein; methylester was found almost exclusively in the A subunit of this heterodimer. The reaction was dependent on the concentration of AdoMet, was constant for 15 min, and increased with increasing calcineurin concentrations. Incorporation was dependent on the ratio of methyltransferase to calcineurin with the steady state of incorporation (0.3–0.4 mol of CH_3 per mol of calcineurin) achieved at a low ratio of methyltransferase/calcineurin increased to ≈2 mol of CH_3 per mol of protein by addition of more methyltransferase. Since this enzyme has a low turnover number [estimated at 0.1 min^{-1} or less (33)], increasing the total enzyme amount would be expected to compensate for the slow rate of the reaction. A detailed kinetic analysis of the carboxyl methylation of calcineurin was judged inappropriate, since the required enzyme concentration was in great excess, compared to substrate, and multiple sites of modification would preclude straightforward interpretation of reaction rates. It was not possible in this study to determine if attachment of the first methyl group alters the rate of the attachment of the second molecule, a possibility that complicates simple kinetic approaches. Thus, a value of 1 mol of CH_3 per mol of calcineurin represents a population average consisting of none, 1, and 2 mol of CH_3 per mol of calcineurin.

Calcineurin carboxyl methylesters were labile in both native and acid-precipitated states, and the half-lives varied with the pH of the reaction. This was especially true of the native form, the half-life of which decreased dramatically above pH 7. Other investigators (14, 32) have examined the chemical stability of the methylester bond. When erythrocyte membrane substrates were examined, three different stability classes were identified ($t_{1/2}$ ≃ 3, 30, and 360 min, respectively) (14). When fractions of carboxyl methylated brain cytosol were examined, two distinct stability classes were observed ($t_{1/2}$ ≃ 1.8 and 17.3 min, respectively) (32). Both of these studies used acid-precipitated protein; however, in the current study, the decay of methylesters on native calcineurin could be resolved into two components, suggesting a labile site of methylation and a more stable site that was resistant to hydrolysis. Such considerations underscore an inherent difficulty in analysis of carboxyl methylation studies—that is, the hydrolysis of formed methylesters may occur at rates comparable to that of the methylation itself, accounting, in part, for the observed substoichiometric modification of proteins.

§In this and other experiments, longer exposure times were necessary to detect any labeling of the B subunit. Estimates from densitometry indicate that >95% of radioactivity was localized to the A subunit.

¶When assayed with 5 mM $MgCl_2$ (instead of Mn^{2+}), calmodulin stimulation was 8- to 10-fold while total activity was reduced by a factor of ≈3. Measurement of Mn^{2+}-stimulated phosphate activity was chosen to examine the effect of modification on intrinsic enzyme activity.

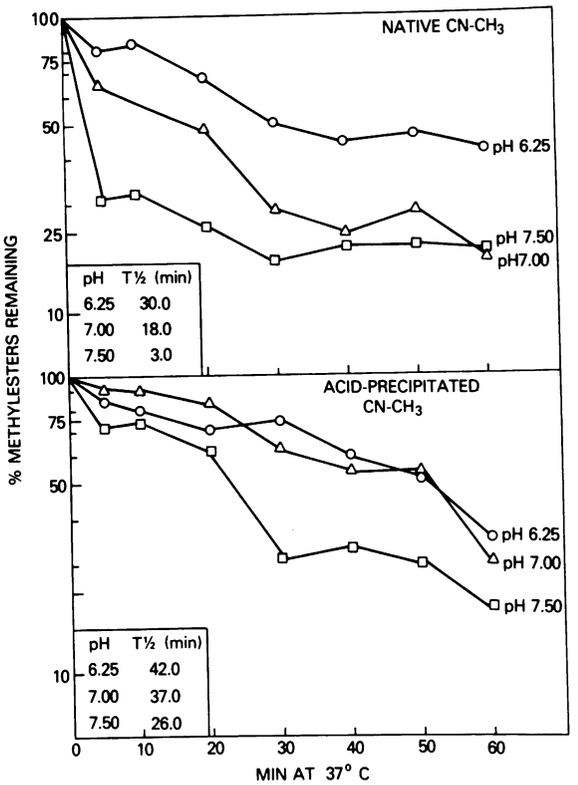

FIG. 3. Stability of carboxyl methylesters on calcineurin. Calcineurin (CN) was first carboxyl methylated with 5 μM Ado[^{3}H]Met and 4 μM bovine brain protein carboxyl *O*-methyltransferase. (*Upper*) For native CN-CH_3, the reaction proceeded for 15 min at 30°C and was inhibited by addition of excess AdoHcy. The pH of the reaction mixture was then adjusted to the listed pH by adding (final concentration) 150 mM Hepes (pH 7.5 and pH 7.0) or 150 mM Mes (pH 6.25) in a final volume of 200 μl. Aliquots were removed at specified times, and trichloroacetic acid-precipitable radioactivity was determined. Half-lives were estimated directly from the graph. (*Lower*) Calcineurin was carboxyl methylated as in the previous experiment. After 15 min at 37°C, the reaction was terminated by addition of 10% tricholoracetic acid and 100 μg of bovine serum albumin. After extensive washes, the pellet was resuspended in either 150 mM Hepes (pH 7.5 and pH 7.0) or 150 mM Mes (pH 6.25), and aliquots were withdrawn at specified times for determination of acid-precipitable radioactivity. Half-lives were calculated from regression analyses of all points. Each experiment was performed twice.

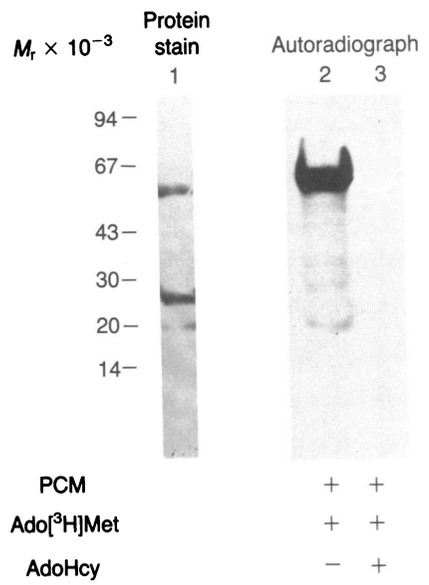

FIG. 4. Acidic NaDodSO_4/PAGE of carboxyl-methylated calcineurin. Calcineurin (4.0 μg), bovine brain protein carboxyl *O*-methyltransferase (4.0 μg; PCM), and 5.0 μCi of Ado[^{3}H]Met (62 Ci/mmol) were incubated in 50 mM Mes (pH 6.25) for 15 min at 37°C. The reaction was terminated by addition of an acidic (pH 2.4) NaDodSO_4-stop solution and subjected to electrophoresis at 40 V. The gel was stained with Coomassie blue and prepared for fluorography. The autoradiograph was exposed for 36 hr at −80°C. Lane 1, protein staining pattern, with the A subunit of calcineurin (M_r = 61,000), protein carboxyl *O*-methyltransferase (M_r = 27,000), and the B subunit of calcineurin (M_r = 18,000). Lane 2, autoradiograph pattern obtained after carboxyl methylation. Lane 3, absence of labeling when 100 μM AdoHcy was included in the reaction.

Other substrates for protein carboxyl *O*-methyltransferase have been examined and found to be substoichiometrically methylated. Calmodulin was demonstrated to be carboxyl methylated (16) but stoichiometry was consistently low. When purified bovine brain Ca^{2+}/calmodulin-dependent phosphodiesterase was carboxyl methylated, ≈20% of the molecules were carboxyl methylated, and the Ca^{2+}/calmodulin-stimulated (but not basal) phosphodiesterase activity was significantly attenuated (20). In that study, the proteins from rat brain that were eluted from calmodulin-Sepharose had a substantial capacity to be carboxyl methylated, which led us to examine calcineurin, the major brain calmodulin-binding protein, in greater detail. To our knowledge, a substrate of protein carboxyl *O*-methyltransferase that has been stoichiometrically methylated has not been reported previously. Whether this can be attributed to higher stability of specific carboxyl methylester environments is unclear at present.

When phosphatase activity was determined after carboxyl methylation, a marked reduction in calmodulin-stimulated activity was noted. Although in two of three experiments the Mn^{2+}-supported activity slightly increased after carboxyl methylation, the effect on calmodulin stimulation was consistently inhibitory, suggesting that carboxyl-methylated calcineurin may have altered regulation by calmodulin. This inhibition of calmodulin-dependent phosphatase activity occurred when 1–2 mol of CH_3 was transferred per mol of calcineurin. Examination of the activity of preparations with lower degrees of modification was not carried out since such samples would clearly contain unmodified, as well as modified, enzyme species. In this study, assays were performed at saturating substrate concentrations to ensure maximal velocities and comparisons were made with the Mn^{2+}- dependent activity, which comprised 30–40% of total calmodulin-supported activity. Since the Mn^{2+}-dependent component was not greatly affected by modification, it seems unlikely that the effects reported are due to denaturation of enzyme activity. Furthermore, calmodulin-dependent phosphatase activity was not directly inhibited by either AdoHcy or AdoMet. Although the phosphatase activity at pH 6.5 was ≈25% of that seen near the pH optimum (≈8.0), stimulation of Mn^{2+}-dependent activity by calmodulin was comparable to that at the higher pH and is comparable to that observed at lower *p*-nitrophenyl phosphate concentrations (29). Thus, the use of this lower pH (6.5) minimized the rapid hydrolysis of carboxyl-methylated calcineurin while retaining the regulatory properties of the enzyme, albeit at a lowered total activity (Fig. 3).

Although it has been suggested that enzyme protein carboxyl *O*-methyltransferase may modify D-aspartate residues forming stable carboxyl methylesters in erythrocytes (10–15), the site(s) of modification, as well as function, may be quite different in brain. Indeed, we have recently localized protein carboxyl *O*-methyltransferase to neurons in specific brain regions (34), suggesting a selective neuronal function in brain, as opposed to a more ubiquitous repair/recognition role proposed for this enzyme in erythrocytes. In addition, we have colocalized both protein carboxyl *O*-methyltransferase and calcineurin in similar neurons of brain (unpub-

Table 1. Effects of carboxyl methylation on the phosphatase activity of calcineurin

Condition	PCM, μM	CH_3/CN, mol/mol	CN activity, μmol/min·mg of CN	% stimulation by calmodulin
		Experiment 1		
Mn^{2+}-stimulated	0	0	1.22 ± 0.40	
Calmodulin-stimulated	0	0	3.70 ± 0.21	206
Mn^{2+}-stimulated	9.1	1.01	0.90 ± 0.21	
Calmodulin-stimulated	9.1	1.01	1.29 ± 0.22	43
		Experiment 2		
Mn^{2+}-stimulated	0	0	1.13 ± 0.06	
Calmodulin-stimulated	0	0	2.54 ± 0.55	126
Mn^{2+}-stimulated	9.1	1.14	1.85 ± 0.16	
Calmodulin-stimulated	9.1	1.14	1.92 ± 0.25	4
		Experiment 3		
Mn^{2+}-stimulated	0	0	1.07 ± 0.19	
Calmodulin-stimulated	0	0	2.63 ± 0.03	146
Mn^{2+}-stimulated	13.5	1.92	1.29 ± 0.14	
Calmodulin-stimulated	13.5	1.92	1.60 ± 0.19	24

Calcineurin (CN) phosphatase activity was measured by using *p*-nitrophenyl phosphate as a substrate, and incorporation of methyl groups was determined in parallel samples. Each point was performed in quadruplicate and represents the mean ± SEM. PCM, protein carboxyl *O*-methyltransferase.

lished data). The present study may suggest a possible regulatory function for protein carboxyl methylation in brain—namely, the control of calmodulin sensitivity of calmodulin-binding proteins. It is also possible that methyltransferase binding to calcineurin directly results in reduced stimulation by calmodulin. Chromatographic experiments suggested that the methyltransferase does not bind to calmodulin-Sepharose (20) nor does it bind to calmodulin-binding proteins immobilized on calmodulin-Sepharose (unpublished data). However, this protein–protein interaction that accompanies modification by the transferase may alter accessibility of the proteins to calmodulin. The high concentration of methyltransferase (≈1–5 μM) and its apparent colocalization with calcineurin in specific neurons may suggest that compartmentalization plays a specific role in regulating activity. Certainly, this may facilitate enzyme modification by the methylase, the *in vivo* activity of which appears quite low. We have now shown that carboxyl methylation of phosphodiesterase (20) and calcineurin reduces their calmodulin-stimulated activities; whether this can be extended to other calmodulin-binding proteins/enzymes or whether this can occur under physiologic conditions is not known. Examination of the physical properties of carboxyl-methylated calcineurin and its interaction with calmodulin may provide clues as to the mechanism of enzymatic activation.

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