Isolation and Kinetic Characterization of the Calmodulin Methyltransferase from Sheep Brain†

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ABSTRACT: The methyltransferase that catalyzes the trimethylation of lysine 115 in calmodulin has been purified from sheep brain. The enzyme is a monomer with an apparent molecular weight of 38 000 on the basis of gel filtration chromatography and SDS–polyacrylamide electrophoresis. In the presence of calcium the methyltransferase exhibited a K_m of 100 nM for unmethylated calmodulin and a k_{cat} of 0.0278 s^{-1}. The enzyme was able to use calcium-depleted calmodulin as a substrate, albeit with less efficiency. The methylation of calcium-depleted calmodulin was inhibited by increases in ionic strength, whereas methylation of calcium-saturated calmodulin was not affected. This suggests a difference in the mode of interaction of calcium-saturated and calcium-depleted calmodulins with the enzyme. As with calmodulin's interactions with other calmodulin-dependent enzymes, the oxidation of the methionines of calmodulin by performic acid treatment decreases the ability of the methyltransferase to recognize and methylate calmodulin. A calmodulin-binding peptide based on the calmodulin-dependent protein kinase II sequence and the naphthalenesulfonamide W-7 inhibit the calmodulin methyltransferase–calmodulin interaction in a calcium-dependent manner. Removal of the NH_2-terminal lobe (residues 1–77) does not affect the ability of the calmodulin methyltransferase to recognize and methylate lysine 115. Thus, the determinants for calmodulin methyltransferase binding reside solely in the COOH-terminal lobe of calmodulin. Further, structural features within this region, in particular, the hydrophobic cleft, that are manifested upon calcium binding may contribute to the interaction of calmodulin with the enzyme.

Calmodulin, a ubiquitous calcium-modulated protein, is commonly found to be trimethylated at lysine 115 [reviewed in Roberts et al. (1986a), Roberts and Harmon (1992), and Siegel et al. (1990)]. A methyltransferase activity that is responsible for the trimethylation of lysine 115 has been demonstrated in numerous tissues and has been partially purified and characterized (Rowe et al., 1986; Roberts et al., 1986b; Oh & Roberts, 1990). Several observations suggest that this enzyme exclusively methylates lysine 115 of calmodulin and that it has a specific recognition site that is dictated by residues adjacent and/or distal to position 115. First, the only documented substrate for the enzyme is calmodulin, and other commonly methylated protein substrates are not recognized by the enzyme (Morino et al., 1987). Second, the levels of the enzyme and endogenous calmodulin methylation appear to be coordinately regulated *in vivo* (Rowe et al., 1986). Third, site-directed mutants of calmodulin in which arginine and isoleucine replace lysine 115 are no longer methylated but are potent competitive inhibitors of the enzyme, suggesting that the recognition site for the enzyme remains intact (Oh & Roberts, 1990; Roberts et al., 1990). Last, studies on a *Paramecium* calmodulin mutant with a substitution of a threonine residue for isoleucine 136 within the fourth calcium-binding loop results in reduced endogenous levels of calmodulin methylation that may reflect a lowered ability of the enzyme to recognize the mutant calmodulin (Lukas et al., 1989). The high specificity and widespread distribution of this enzyme suggests that it coevolved with calmodulin as a dedicated methyltransferase.

Calmodulin methylation of lysine 115 affects certain *in vitro* calmodulin activities, including NAD kinase activation (Roberts et al., 1986b, 1990) and susceptibility of calmodulin to ubiquitination (Gregori et al., 1985, 1987). These effects, along with the observation that calmodulin methylation is subject to developmental regulation (Oh & Roberts, 1990; Oh et al., 1992), suggest that lysine methylation could play a role in the regulation of calmodulin activity and homeostasis. This is supported by transgenic plant studies that show that the introduction of calmodulin mutants with substitutions at position 115 that are incapable of methylation result in altered plant growth properties (Roberts et al., 1992).

To increase our understanding of how the calmodulin methyltransferase[1] recognizes and interacts with calmodulin with high specificity, we have purified the enzyme from sheep brain and have investigated its steady-state kinetic properties under conditions that affect calmodulin structure.

MATERIALS AND METHODS

Materials. [^{3}H]AdoMet,[2] 60–85 Ci/mmol, was purchased from New England Nuclear. Phenyl-Sepharose CL-4B,

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[1] Calmodulin-lysine *N*-methyltransferase, EC 2.1.1.60.

[2] Abbreviations: AdoMet, *S*-adenosylmethionine; [^{3}H]AdoMet, [*methyl*-^{3}H]-*S*-adenosyl methionine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; VU-1 calmodulin, calmodulin derived from a cloned synthetic gene (Roberts et al., 1985); W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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cyanogen bromide activated Sepharose 4B, and Superose HR 12 were purchased from Pharmacia Fine Chemicals. Phosphocellulose P-11 was obtained from Whatman Biochemical. Calmodulin-dependent protein kinase II synthetic peptides were purchased from Bachem California. W-7 was purchased from Fluka Biochemicals. Sheep brains were obtained from the Food Technology Department of the University of Tennessee. Bovine brain calmodulin–Sepharose 4B was prepared by the method of Klee and Krinks (1978). Other reagents were purchased from commercial sources and were of reagent grade or better.

Preparation of Calmodulins and Calmodulin Fragments. Recombinant DNA derived VU-1 calmodulin expressed in *Escherichia coli* was prepared as described previously (Roberts et al., 1985, 1987). Preparation of performic acid oxidized VU-1 calmodulin was done essentially as described by Van Eldik and Watterson (1981). Performic acid reagent was prepared by adding 0.5 mL of 30% (v/v) hydrogen peroxide to 9.5 mL of formic acid and incubating the resulting solution at room temperature for 2 h. The reagent was chilled on ice for 30 min prior to use. VU-1 calmodulin (220 μg) was dissolved in 1 mL of formic acid and incubated on ice for 30 min. Three milliliters of the performic acid reagent was added, and the reaction was allowed to proceed for 150 min on ice. Five hundred microliters of 10 mM ammonium bicarbonate was added, and the mixture was dialyzed against 10 mM ammonium bicarbonate at 4 °C overnight. The sample was lyophilized and redissolved in 50 μL of 10 mM ammonium bicarbonate. The final product was stored at −80 °C.

A tryptic fragment (amino acid residues 78–148) that contains the COOH-terminal lobe of VU-1 calmodulin was prepared by a modification of the method of Newton et al. (1984). VU-1 calmodulin (2 mg) was incubated with trypsin (32 μg) at 30 °C for 40 min in 1 mL of 50 mM NH_4HCO_3, 50 mM NaCl, and 2 mM $CaCl_2$. The digestion reaction was terminated by adding 100 μg of soybean trypsin inhibitor. The COOH-terminal fragment of VU-1 calmodulin was purified by HPLC on a Vydac phenyl reverse-phase column (0.46 cm × 15 cm) as described by Newton et al. (1984). There is only one tyrosine in VU-1 calmodulin, at position 138 (Roberts et al., 1985). Thus, the tyrosine-containing COOH-terminal fragment and the NH_2-terminal fragment were distinguished by their differential absorbance at 280 nm. Peak fractions were collected, lyophilized, and resuspended in 500 μl of deionized water. The sample was purified further by FPLC on a Mono-Q column (0.5 × 5 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.2 mM EGTA, and 0.05% (w/v) CHAPS. The COOH-terminal fragment was eluted with a linear gradient of 0–0.5 M NaCl in equilibration buffer. The column was run at a flow rate of 0.5 mL/min, and the column effluent was monitored at 280 nm. The peak fractions were collected, and the purity of the final product was verified by SDS–PAGE. The final product was stored at −80 °C until assay.

Purification of Calmodulin-Lysine N-Methyltransferase. Calmodulin methyltransferase was purified from sheep brains by using a modification of the procedure of Oh and Roberts (1990). Ten sheep brains (856 g) were homogenized with 3.5 vol of 25 mM HEPES–NaOH (pH 7.4), 4 mM β-mercaptoethanol, 0.25 M sucrose, 5 mM EGTA, and 0.1 mM PMSF. The homogenate was centrifuged at 11000*g* for 30 min. The supernatant fraction was removed and stored on ice, while pellets were reextracted in 1 vol of homogenization buffer and centrifuged as described above. The supernatant fractions were combined and were subjected to differential ammonium sulfate fractionation (Rowe et al., 1986). The pellet obtained from the 35% to 70 % precipitation step was resuspended in 25 mM HEPES–NaOH, pH 6.8, 0.1 mM EGTA, 4 mM β-mercaptoethanol, and 0.1 mM PMSF and dialyzed against the same buffer overnight at 4 °C. The dialyzed sample was centrifuged at 15000*g* for 30 min at 4 °C. The supernatant fraction was combined with 600 mL of phosphocellulose resin which was equilibrated in phosphocellulose column buffer [25 mM HEPES–NaOH, pH 6.8, 4 mM β-mercaptoethanol, 0.01% (w/v) Triton X-100, and 0.1 mM PMSF]. The suspension was mixed for 90 min at 4 °C, and the unadsorbed fraction was collected by vacuum filtration. The resin was washed with 4 L of phosphocellulose column buffer and was repacked into a column (6 x 20 cm). The column was washed until the A_{280} of the effluent was zero. The enzyme was eluted with 25 mM HEPES–NaOH, pH 6.8, 4 mM β-mercaptoethanol, 0.01% (w/v) Triton X-100, 0.1 mM PMSF, and 0.5 M NaCl. The enzyme was concentrated by ultrafiltration in an Amicon stirred cell with a YM-10 membrane. The sample was dialyzed overnight against 4 L of calmodulin–Sepharose buffer [20 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.1 mM $CaCl_2$, 0.05% (w/v) CHAPS, 4 mM β-mercaptoethanol, and 0.1 mM PMSF]. CHAPS was included in all buffers at this point since it was found to stabilize and increase the recovery of enzyme activity during these later purification steps (data not shown). The sample was applied to a bovine calmodulin–Sepharose column (1.5 × 4 cm), and the column was washed with 100 mL of calmodulin–Sepharose buffer containing 2 mM DTT instead of β-mercaptoethanol. The column was then washed with 100 mL of calmodulin–Sepharose buffer with 0.6 M NaCl and was eluted with 20 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM EGTA, 1 M NaCl, and 0.05% (w/v) CHAPS. The fractions with calmodulin methyltransferase activity were pooled and concentrated by ultrafiltration on a Centricon-30 unit (Amicon).

The calmodulin–Sepharose-purified enzyme was purified further by molecular exclusion FPLC on a Superose HR 12 column (1 × 30 cm) in 25 mM HEPES–NaOH, pH 7.5, 2 mM DTT, 0.2 M NaCl, and 0.05% (w/v) CHAPS. Fractions that tested positive for calmodulin methyltransferase activity were pooled and were stored at −80 °C. The enzyme activity was stable for several months stored in this manner.

Calmodulin-Lysine N-Methyltransferase Kinetics. Methyltransferase activity was assayed by a modification of the protocol of Rowe et al. (1983) as described by Oh and Roberts (1990). The standard reaction mixture (100 μl) contained VU-1 calmodulin (1.67 μg) and 12.0 μM [^{3}H]AdoMet (1.5 μCi) in methyltransferase reaction buffer [0.1 M glycylglycine–NaOH, pH 8.0, 0.15 M KCl, 2 mM $MgCl_2$, 5 mM DTT, 0.01% (w/v) Triton X-100, and 0.1 mM $CaCl_2$]. Reactions were terminated by heating at 90 °C for 3 min, and labeled calmodulin was fractionated on phenyl-Sepharose and analyzed as previously described (Oh & Roberts, 1990). For determinations of the dependence of the enzyme activity on calcium, the assay buffer was modified to contain Chelex-treated 25 mM HEPES–NaOH, pH 7.5, and concentrations of EGTA, $CaCl_2$, and $MgCl_2$ to yield 5 mM free Mg^{2+} and a free Ca^{2+} concentration that varied from 10^{-8} to 10^{-4} M.

A modified protocol was used for the assay of the calmodulin methyltransferase in the presence of calmodulin-binding peptides. Assays were performed under the standard calmodulin methyltransferase assay conditions in the presence of 0.1 mM $CaCl_2$ and 1 μM VU-1 calmodulin. After termination of the reaction, 10 μl of 10 mM EGTA was added and the peptide was removed by adsorption to 40 μl of

phosphocellulose. The unadsorbed fraction, which contains calmodulin, was then analyzed by the standard phenyl-Sepharose method described above.

Previous kinetic analyses of the methyltransferase suggested a sequential bireactant mechanism (Oh, 1992). Thus, the analysis of kinetic data and the determination of kinetic parameters in the present study were done by using the form of the Michaelis–Menten equation for sequential bireactant systems:

$$V = \frac{V_{max}[A][B]}{[A]K_{mB} + [B]K_{mA} + [A][B]}$$

where A and B are the varied substrates and K_{mB} and K_{mA} are the Michaelis–Menten constants for substrates A and B, respectively.

General Methods. For estimations of the native molecular weight of the calmodulin methyltransferase, the purified enzyme was mixed with gel filtration protein standards (Bio-Rad) and was applied to a Superose HR 12 column (1.0 × 30 cm). Fractions (0.25 mL) were collected and assayed under standard assay conditions as described above. SDS–polyacrylamide gel electrophoresis was done by using the method of Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976) or by the bicinchoninic acid method of Smith et al. (1985).

RESULTS

Purification of the Calmodulin Methyltransferase. In previous work, we developed a protocol for the partial purification of the calmodulin methyltransferase from sheep brain (Oh & Roberts, 1990). The protocol was improved in the present study, and by multiple steps, including calmodulin–Sepharose affinity chromatography and chromatography on Superose HR 12, the enzyme was purified over 20 000-fold compared to the activity of the starting extract and possessed a final specific activity that ranged from 20 to 50 nmol of methyl groups incorporated/min/mg of protein. The purified enzyme eluted from the Superose column with an apparent molecular weight of 38 000 (Figure 1A) and exhibited a single major band with an apparent molecular weight of 38 000 after SDS–polyacrylamide electrophoresis (Figure 1B). Variable amounts of a minor band with an apparent molecular weight of 35 000 were observed in some preparations. The data suggest that the sheep brain calmodulin methyltransferase is a monomer of molecular weight 38 000. This preparation was used for the experiments pursued in the present study.

Calcium Dependence of the Calmodulin–Calmodulin Methyltransferase Interaction. To investigate the influence of calcium on the interaction of calmodulin with the methyltransferase, affinity chromatography of the enzyme on calmodulin–Sepharose was done under various conditions (Figure 2). In a low ionic strength buffer (Figure 2A), the enzyme was bound to the resin in the presence of either calcium or EGTA, suggesting a calcium-independent interaction. However, when chromatography was conducted at a higher salt concentration (Figure 2B), the enzyme was bound by calmodulin–Sepharose in a calcium-dependent fashion and could be quantitatively eluted from the resin by the addition of EGTA.

The calcium-dependent interaction of the methyltransferase with calmodulin as a function of ionic strength is also manifested in activity measurements. In the presence of 100 μM $CaCl_2$ the enzyme showed little sensitivity to the change of KCl concentration (Figure 3A). However, enzyme activity was decreased in an ionic strength dependent manner in the

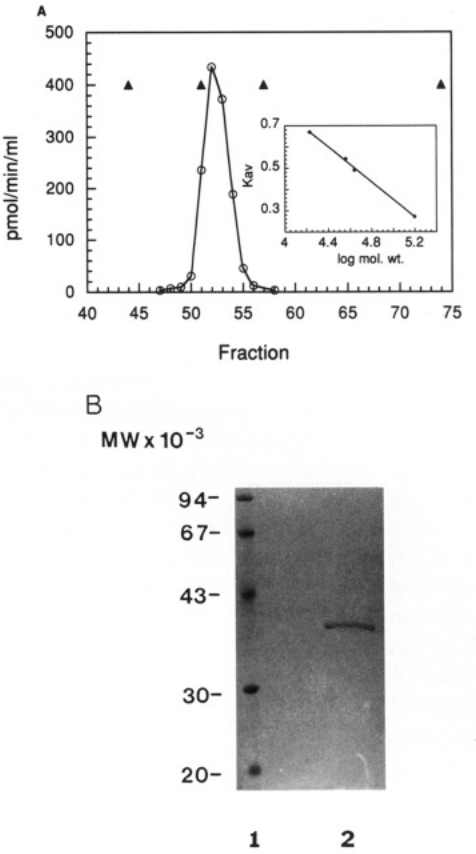

FIGURE 1: Molecular weight analysis of purified calmodulin *N*-methyltransferase from sheep brain. (A) Calmodulin *N*-methyltransferase was subjected to FPLC chromatography on a Superose HR 12 column (1.0 × 30 cm). Fractions (0.25 mL) were collected at a flow rate of 0.2 mL/min. Filled triangles represent the peak elution positions of gel filtration standards: 158 000, γ-globin; 44 000, ovalbumin; 17 000, myoglobin; 1,350, cyanocobalamin. Open circles represent the calmodulin methyltransferase activity (pmol/min/mL) of each fraction. The inset shows the plot of K_{av} vs log(mol wt). (B) SDS-PAGE of purified calmodulin *N*-methyltransferase. Calmodulin *N*-methyltransferase purified through the Superose step was analyzed by electrophoresis on a 12.5% (w/v) SDS–polyacrylamide gel. Lane 1, molecular weight standards; Lane 2, purified calmodulin *N*-methyltransferase.

presence of 1 mM EGTA (Figure 3A). These data support the affinity chromatography results and suggest that the interaction of the enzyme with calmodulin shows a calcium dependence that is a function of ionic strength. For kinetic analyses we adopted a standard concentration of ions (0.15 M KCl and 5 mM $MgCl_2$) that approximated physiological conditions. In the presence of calcium, the K_m value for calmodulin and the k_{cat} value were 100 nM and 0.0278 s^{-1} (Table I). However, in the presence of EGTA the methyltransferase showed a lower enzyme efficiency, reflected by a k_{cat}/K_m ratio that was 6-fold lower than that observed in the presence of calcium (Table I).

Under standard conditions, calcium stimulates the methyltransferase activity 7-fold and shows a $K_{0.5}$ of 3 μM (Figure 3B). This value is within the range of K_d values reported for calcium binding to calmodulin [reviewed in Klee and Vanaman (1982)]. Further, calcium-binding experiments show no interaction between calmodulin methyltransferase and calcium (data not shown). Thus, the effect of calcium on the methyltransferase activity appears to be through the binding of calcium to calmodulin rather than through a direct effect of calcium on the enzyme. Overall, the results suggest that the enzyme prefers the calcium-bound form of calmodulin as a substrate. This is consistent with previous observations that

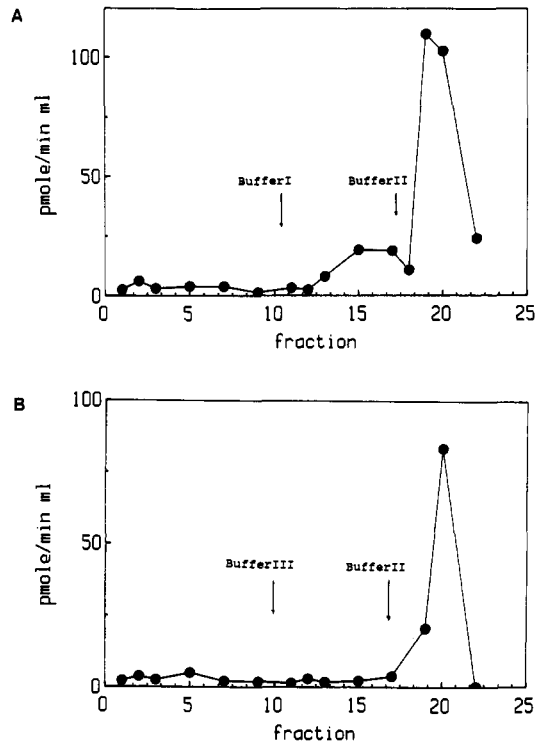

FIGURE 2: Calcium-dependent interaction of calmodulin *N*-methyltransferase with calmodulin–Sepharose. Calmodulin–Sepharose columns (0.74 × 2.3 cm) were equilibrated with 20 mM Tris-HCl and 0.1 mM $CaCl_2$, pH 7.4. Samples (225 μg of protein) were applied. The columns were washed with 10 mL of equilibration buffer and were eluted under the following conditions; 1-mL fractions were collected. (A) Buffer I, 20 mM Tris-HCl and 1 mM EGTA, pH 7.4; buffer II, 20 mM Tris-HCl, 1 mM EGTA, and 0.6 M NaCl, pH 7.4. (B) Buffer III, 20 mM Tris-HCl, 0.6 M NaCl, and 0.1 mM $CaCl_2$, pH 7.4; buffer II, 20 mM Tris-HCl, 1 mM EGTA, and 0.6 M NaCl, pH 7.4.

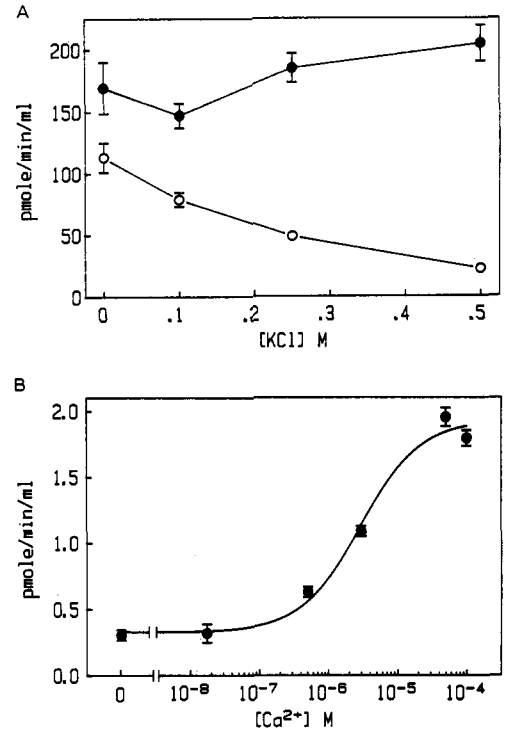

FIGURE 3: Effect of calcium on calmodulin *N*-methyltransferase activity. (A) Calcium dependence of the methyltransferase activity as a function of ionic strength. Assays were performed under the standard assay conditions in the presence of 0, 100, 250, and 500 mM KCl with 100 μM $CaCl_2$ (●) or 1 mM EGTA (○). The data points represent the mean of four determinations with error bars showing the standard error of the mean. (B) Calcium titration of the methyltransferase activity. Activity was measured over the range of calcium concentrations shown by using an EGTA/Ca^{2+} buffering system. The data points represent the mean of three determinations with the error bars showing the standard error of the mean.

show that mutations in the calcium-binding sites of calmodulin can lead to lower endogenous levels of methylation (Lukas et al., 1989).

Effect of Methionine Oxidation of Calmodulin on Its Interaction with Methyltransferase. The finding that the calmodulin methyltransferase interacts with calmodulin in a calcium-dependent fashion raises the question of whether similarity exists between the methyltransferase and other calmodulin-dependent enzymes in their mode of interaction with calmodulin. On the basis of the crystal structure of calcium-saturated calmodulin (Babu et al., 1988), nearly all methionine residues reside in the two hydrophobic clefts formed in the NH_2- and COOH-terminal lobes. These hydrophobic clefts become exposed upon the binding of calcium and are directly involved in the binding of calmodulin-dependent enzymes and calmodulin-antagonistic drugs. The importance of methionine residues within these regions for the interaction of calmodulin with calmodulin-dependent enzymes is reflected by the extreme sensitivity of calmodulin activity to oxidizing agents that modify methionine residues (Walsh & Stevens, 1978). As shown in Figure 4, performic acid oxidization of calmodulin dramatically decreased its ability to serve as a substrate for the calmodulin methyltransferase, even in the presence of calcium. Thus, as with calmodulin's interactions with calmodulin-regulated enzymes, the integrity of the methionine residues of calmodulin is essential for its interaction with the methyltransferase.

Inhibition of Calmodulin Methylation by Calmodulin-Binding Drugs and Peptides. A synthetic peptide (LKKFNARRKLKGAILTTMLA, residues 290–309), corresponding to the calmodulin binding domain peptide of calmodulin-dependent protein kinase II, and the naphthalenesulfonamide derivative W-7 were tested for their ability to inhibit calmodulin methylation. In the presence of 1 μM VU-1 calmodulin and 100 μM Ca^{2+}, the calmodulin-binding peptide inhibited calmodulin *N*-methyltransferase activity with an IC_{50} of 570 nM (Figure 5A). This is consistent with a 1:1 titration of peptide and calmodulin that is expected on the basis of the concentration of calmodulin (1 μM) and the low K_d (45 nM) for the interaction of peptide with calmodulin (Meyer et al., 1992). The peptide did not affect the rate of calmodulin methylation in the absence of calcium (data not shown). Thus, the inhibition appears to be due to the calcium-dependent interaction of the peptide with calmodulin rather than a direct influence on the methyltransferase. No inhibition

Table I: Catalytic Parameters of Calmodulin *N*-Methyltransferase[a]

parameter[b]	in the presence of 100 μM $CaCl_2$	in the presence of 1 mM EGTA
K_m for calmodulin (nM)	100	220
V_{max} (nmol/min/mg of protein)	44.1	16.7
k_{cat} (s^{-1})	0.0278	0.0106
k_{cat}/K_m (s^{-1} M^{-1})	27.8×10^4	4.82×10^4

[a] Assays were performed under standard conditions and in the presence of 0.15 M KCl. [b] Data for the calculation of K_m and V_{max} were derived from double-reciprocal plots of three assay sets using calmodulin as the variable substrate and maintaining AdoMet at constant concentrations of 2.4, 4.8, and 12.1 μM. K_m and V_{max} were obtained from the replots of 1/[AdoMet] vs $\text{slope}_{1/[CaM]}$ and 1/[AdoMet] vs $1/V_{max_{app}}$, respectively. k_{cat} was calculated using 38 000 as the molecular weight of the enzyme.

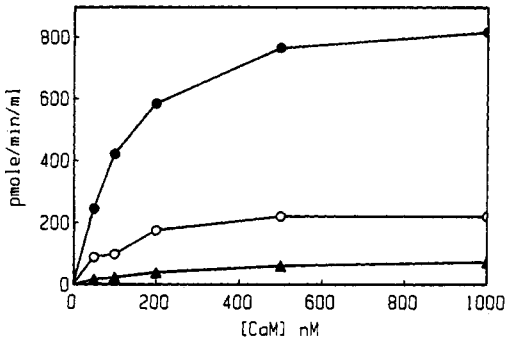

FIGURE 4: Effect of methionine oxidation on the kinetics of lysine methylation of VU-1 calmodulin. Calmodulin methylation was assayed under standard conditions with 12 μM AdoMet and with calmodulin as the varied substrate. Substrates: unoxidized VU-1 calmodulin in the presence of 100 μM $CaCl_2$ (●), unoxidized VU-1 calmodulin in the presence of 1 mM EGTA (○), and performic acid oxidized VU-1 calmodulin in the presence of 100 μM $CaCl_2$ (▲).

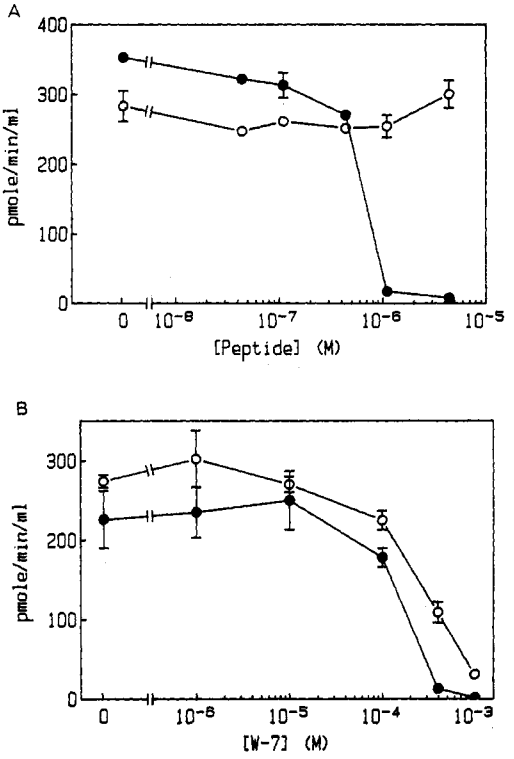

FIGURE 5: Inhibition of calmodulin methyltransferase by calmodulin-antagonistic peptides and drugs. Calmodulin *N*-methyltransferase was assayed under standard conditions in the presence of a constant concentration of AdoMet (12 μM), VU-1 calmodulin or calmodulin fragment (1 μM), and $CaCl_2$ (100 μM). (A) Influence of the calmodulin-binding domain (residues 290–309) of calmodulin-dependent protein kinase II on the methylation of VU-1 calmodulin (●) or the COOH-terminal fragment (residues 78–148) of VU-1 (○). (B) Influence of W-7 on the methylation of VU-1 calmodulin (●) or the COOH-terminal fragment (residues 78–148) of VU-1 (○). Error bars show the standard errors.

was observed with a negative-control peptide, the autophosphorylation domain (residues 281–289) of CaM kinase II, which does not bind calmodulin (data not shown).

The calmodulin antagonist W-7 also inhibits the methylation of calmodulin in a dose-dependent manner (Figure 5B). Similar to the results with the calmodulin-binding peptide, inhibition by W-7 was observed in the presence of calcium but not in the presence of EGTA. Overall, the data suggest that compounds that antagonize the binding of calmodulin-regulated enzymes to calmodulin also disrupt the calmodulin–calmodulin methyltransferase interaction.

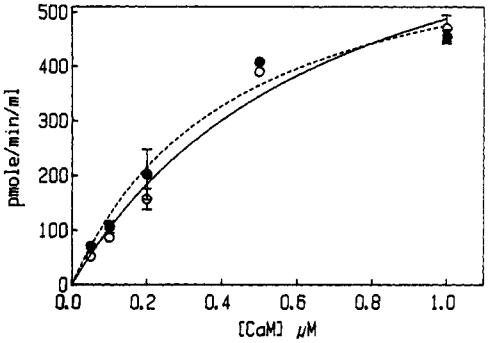

FIGURE 6: Methylation of the COOH-terminal lobe (residues 78–148) of calmodulin. Assays were conducted under conditions identical to those described in Figure 4. Substrates: Filled circles (- - -), normal, uncleaved VU-1 calmodulin; unfilled circles (—), COOH-terminal fragment (residues 78–148) generated from partial trypsin cleavage. Error bars show the standard errors.

Interaction of Calmodulin Methyltransferase with the COOH-Terminal Lobe of Calmodulin. The site of post-translational methylation of calmodulin resides in the loop between the F-helix of the third EF-hand calcium-binding site and the E-helix of the fourth EF-hand calcium-binding site within the COOH-terminal lobe (Babu et al., 1988). The NH_2- and COOH-terminal lobes of calmodulin are both required for the activation of most calmodulin-dependent enzymes since both hydrophobic clefts engage in calmodulin–target protein binding (Ikura et al., 1992; Meador et al., 1992). As a result, fragments that contain either the COOH-terminal or the NH_2-terminal lobe alone are generally poor activators of most calmodulin-dependent enzymes (Drabikowski et al., 1982; Guerini et al., 1984; Kuznicki et al., 1981; Newton et al., 1984; Wall et al., 1981; Walsh et al., 1977). To test whether both lobes are necessary for the binding to the methyltransferase, we isolated the COOH-terminal calcium-binding lobe (residues 78–148) generated by partial tryptic digestion and tested its properties as a methyltransferase substrate. This region was selected since it retains a conformation similar to that of the native protein (Dalgarno et al., 1984) and contains the methylation site. As shown in Figure 6, the rate of methylation obtained with the COOH-terminal lobe is indistinguishable from that of intact VU-1 calmodulin. Thus, the NH_2-terminal lobe of calmodulin (residues 1–77) is not necessary for binding and recognition by the methyltransferase.

We also expanded our studies of the effect of calmodulin-binding peptides and drugs on methylation to include analyses of the COOH-terminal lobe. As shown in Figure 5B, W-7 inhibits the methylation of the COOH-terminal lobe in a manner indistinguishable from that observed with intact VU-1. This is not surprising since the COOH-terminal fragment still possesses one of the hydrophobic clefts and retains the ability to bind calmodulin antagonists (Vogel et al., 1983; Krebs et al., 1984; Newton et al., 1984). However, the calmodulin-binding peptide, which requires both lobes of calmodulin for high-affinity binding (Ikura et al., 1992; Meador et al., 1992), did not inhibit the methylation of the COOH-terminal lobe (Figure 5A). These findings support the data obtained with intact calmodulin and suggest that calmodulin methylation is antagonized by peptides and drugs that bind in a calcium-dependent manner to the hydrophobic cleft formed within the COOH-terminal lobe.

DISCUSSION

Calmodulin *N*-methyltransferase, the enzyme responsible for the trimethylation of lysine 115 of calmodulin, has been

purified from sheep brain. The enzyme is a monomer of molecular weight 38 000 on the basis of gel filtration and SDS–PAGE analyses. Under physiological conditions of ionic strength and pH, the calcium-bound form of calmodulin is the preferred substrate for the enzyme. Similar to most calcium–calmodulin-dependent enzyme activities, the ability of the calmodulin methyltransferase to recognize and methylate calmodulin is sensitive to methionine-oxidizing agents as well as to peptides and drugs that interact with calmodulin in a calcium-dependent manner. However, unlike most calmodulin-dependent enzymes, removal of the NH_2-terminal lobe (residues 1–77) does not affect the ability of the calmodulin methyltransferase to recognize and methylate lysine 115. Overall, the data suggests that determinants for calmodulin methyltransferase binding reside in the COOH-terminal lobe of calmodulin. Further, structural features within this region that are manifested upon calcium binding may contribute to the interaction of calmodulin with the enzyme.

Studies of the calmodulin methyltransferase have indicated that this enzyme is highly specific for lysine 115 and will not methylate other lysine residues on calmodulin (Rowe et al., 1986; Oh & Roberts, 1990) or a variety of other proteins that are commonly methylated on lysine residues (Morino et al., 1987). In the present study we have begun to define the structural determinants that lead to this high degree of specificity. On the basis of the present results, it appears as if the calmodulin methyltransferase recognition site is located within residues 78–148, which contain the two EF-hand structures which constitute the COOH-terminal calcium-binding lobe.

There are four methionines within the COOH-terminal lobe of VU-1 calmodulin (residues 109, 124, 145, and 146). On the basis of the crystal structure of calcium-saturated calmodulin (Babu et al., 1988) all four methionine residues reside in the hydrophobic cleft formed within the COOH-terminal lobe upon calcium binding and are involved in the binding of peptides (O'Neil et al., 1989; Ikura et al., 1992; Meador et al., 1992). The data in the present study suggest that the oxidation of these residues to methionine sulfone by performic acid treatment significantly decreases the ability of the calmodulin methyltransferase to recognize and methylate calmodulin. This finding suggests that the integrity of the hydrophobic cleft is important for the recognition of calmodulin with the methyltransferase. This is supported by previous findings by Lukas et al. (1989) that showed that *Paramecium* mutants that possess a substitution of threonine for isoleucine 136 are incompletely methylated *in vivo*. This residue is in the core of the COOH-terminal hydrophobic cleft (Babu et al., 1988) and is always an aliphatic hydrophobic residue (Kretsinger, 1980; Moncrief et al., 1990). The importance of this region of calmodulin in recognition by the methyltransferase is also supported by the synthetic peptide and W-7 inhibition data since the hydrophobic cleft is involved in the binding of these compounds (Ikura et al., 1992; Meador et al., 1992).

On the basis of the X-ray crystal structure of calmodulin, lysine 115 is exposed to solvent and is found on the opposite side of the COOH-terminal lobe, away from the exposed hydrophobic cleft (Babu et al., 1988). Thus, it is not clear whether the hydrophobic cleft is directly involved in calmodulin methyltransferase binding or whether conformational changes associated with methionine oxidation or calmodulin antagonist binding indirectly influence the calmodulin methyltransferase binding site. On the basis of structural comparisons of calcium–calmodulin and the calcium–calmodulin–peptide complex, the major conformational change associated with peptide binding is the bending of the central α-helix of calmodulin, whereas the conformation of the NH_2- and COOH-terminal calcium-binding lobes is not radically altered (Babu et al., 1988; Ikura et al., 1992; Meador et al., 1992). Since the calmodulin methyltransferase binding site appears to be localized completely within the COOH-terminal lobe, conformational changes associated with peptide or drug binding are probably not involved in the inhibition of methylation by these compounds. Further investigation, particularly with site-directed mutants of calmodulin, will help clarify the role of the hydrophobic cleft in calmodulin methylation.

Although the calmodulin methyltransferase yields higher activity with the calcium-bound form of calmodulin, the calcium-depleted form of calmodulin is also recognized and methylated by the enzyme. However, the interaction of the calcium-depleted form of calmodulin with the methyltransferase is only observed under conditions of low ionic strength. In contrast, the interaction of the methyltransferase with calcium-bound calmodulin shows no ionic strength dependence. This suggests a fundamental difference in the binding of the calmodulin methyltransferase to calcium-bound and calcium-depleted calmodulins. This is supported by the findings of the synthetic peptide and W-7 inhibitory data that show that these compounds are only effective in the presence of calcium. Previous studies (Rowe et al., 1986) have suggested that the calmodulin methyltransferase is a highly basic protein. Thus, ionic interactions of the enzyme and the acidic, negatively charged calmodulin may help to account for the calcium-independent activity observed at low ionic strength.

Although it is clear that calcium enhances the rate of methylation of calmodulin, the physiological significance of this observation is not yet known. While lysine methylation is commonly thought to be an irreversible enzymatic reaction (Paik & Kim, 1990), it is apparent that calmodulin methylation *in vivo* can be a dynamic process that varies depending upon the developmental state of the tissue. For example, Oh and Roberts (1990) and Oh et al. (1992) have observed large changes in the level of calmodulin methylation in plant cells, that depend upon the stage of growth. Rowe et al. (1986) have also reported differences in calmodulin methylation between various animal tissues. While the parameters that regulate calmodulin methylation *in vivo* are poorly defined, the influence of cytosolic calcium on the rate of calmodulin methylation could contribute to the control of methylation.

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