Calmodulin N-Methyltransferase
PARTIAL PURIFICATION AND CHARACTERIZATION*

Paul M. Rowe¢, Lynda S. Wright, and Frank L. Siegel§
From the Departments of Physiological Chemistry and Pediatrics and the Harry A. Waisman Center on Mental Retardation and Human Development, University of Wisconsin Medical School, Madison, Wisconsin 53706

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The distribution, properties, and substrate specificity of S-adenosylmethionine:calmodulin (lysine) N-methyltransferase (EC 2.1.1.60, calmodulin N-methyltransferase) of the rat have been studied. This enzyme is cytosolic and is found at high levels in tissues with high levels of calmodulin and at low levels in tissues with little calmodulin. In liver, heart, and skeletal muscle, which have low levels of calmodulin and very low calmodulin N-methyltransferase activity (a low ratio of calmodulin N-methyltransferase to calmodulin), calmodulin was found to be incompletely methylated, as judged by its ability to act as a substrate for purified calmodulin N-methyltransferase.

Calmodulin N-methyltransferase was purified 470-fold with a 3.5% yield from rat testis cytosol, using ammonium sulfate precipitation and chromatography on DEAE-cellulose, CM-Sepharose, and Sephadex G-100. At pH 7.4, calmodulin N-methyltransferase did not bind to DEAE-cellulose, but bound strongly to CM-Sepharose. The enzyme eluted from Sephadex G-100 with an apparent molecular weight of 55,000. Purified calmodulin N-methyltransferase was incubated with extracts of rat tissues, and [methyl-3H]AdoMet and methylated proteins were resolved by electrophoresis in an attempt to discover substances other than calmodulin, but this enzyme only catalyzed the methylation of calmodulin, indicating a high degree of substrate specificity. Conditions were established for the in vitro preparative methylation of des(methyl)-calmodulin from Dictyostelium discoideum. Three moles of methyl/mol of calmodulin were incorporated into lysine 115 of des(methyl)calmodulin, resulting in the formation of 1 mol of trimethyllysine at the site normally methylated in calmodulins from most species. Activation of cyclic nucleotide phosphodiesterase by des(methyl)calmodulin was indistinguishable from activation by in vitro methylated or sham methylated Dictyostelium calmodulin, indicating that methylation does not affect the ability of calmodulin to activate this enzyme.

Calmodulin is a multifunctional high-affinity calcium-binding regulatory protein found in all eucaryotes (1, 2). Its amino acid sequence is highly conserved and includes a single residue of ε-N-trimethyllysine at position 115 in most species (1, 3). Lysine residue 115 of calmodulin is methylated post-translationally by S-adenosylmethionine:calmodulin (lysine) N-methyltransferase (EC 2.1.1.60; calmodulin-lysine methyltransferase); a previous report from this laboratory demonstrated this activity in rat brain (4). In that study we employed a single-step chromatographic procedure to separate calmodulin N-methyltransferase from calmodulin. More recently, we have developed two radiometric assays for calmodulin N-methyltransferase; both utilize the naturally non-N-methylated calmodulin from Dictyostelium discoideum as substrate (5, 6).

Although the biological functions subserved by protein N-methylation are not known, protein (lysine) N-methyltransferases from several sources have been characterized; for reviews, see Refs. 7 and 8. Cytochrome c (lysine) N-methyltransferases have been isolated from Neurospora crassa (9), Saccharomyces cerevisiae (10), and wheat germ (11). Riboosomal proteins from both procaryotes and eucaryotes contain methylated lysine residues (7), and a ribosomal protein lysine N-methyltransferase has been isolated from Escherichia coli (12). Histones from most cells contain methylated lysine residues (7), and two histone (lysine) N-methyltransferases from mammalian sources have been partially purified (7, 13, 14). These studies indicated that each of the protein (lysine) N-methyltransferases has a high degree of substrate specificity.

N-Methylated and non-N-methylated calmodulins have similar abilities to activate cyclic nucleotide phosphodiesterase (15, 16), but the maximal activation of pea seedling NAD kinase by non-N-methylated calmodulins is 3–4-fold greater than the maximal activation of this enzyme by N-methylated calmodulins (15, 16). Although the primary structure of the methylated and nonmethylated calmodulins from various species is similar, it is in no case identical, so that differences in activity between these proteins could result from differences other than the presence or absence of trimethyllysine. One way to control for these amino acid sequence differences would be to compare a nonmethylated calmodulin with calmodulin from the same species which had been methylated in vitro.

In this report we present quantitative data on the subcellular and tissue distribution of calmodulin N-methyltransferase, and we relate the tissue distribution of this enzyme to that of calmodulin; evidence is presented for the presence of incompletely methylated calmodulin in some rat tissues. The partial purification of calmodulin N-methyltransferase is described, as is further evidence of its substrate specificity. We have used the mammalian enzyme to methylate des(methyl)-calmodulin from D. discoideum and have determined the
stochiometry and site of this modification. Methylation and nonmethylated calmodulins were compared with respect to their abilities to activate cyclic nucleotide phosphodiesterase.

**MATERIALS AND METHODS**

**Chemicals and Organisms—**Holtzman rats were raised in our breeding colony. *D. discoideum* (strain AX-2) is grown as described (6). Enzymatic assays were prepared by the method of Egrie and Siegel (20). All assays were done under conditions where enzyme activity is constant with time and proportional to enzyme concentration. Briefly, reactions were carried out in a volume of 100 µl, using as substrates [methyl-3H]AdoMet (10 µM) and purified Dicyostelium calmodulin (8 µg/tube for the assay of cyclic nucleotide N-methyltransferase activity, or 0.05 µg/tube for the assay of partially purified calmodulin N-methyltransferase activity). Subcellular distribution studies were analyzed by an acid precipitation method (5); all other assays used the phenyl-Sepharose chromatographic method (6). In assays of partially purified calmodulin N-methyltransferase, the incubation buffer was modified to contain (final concentrations) 0.1 M glycylglycine-NaOH, pH 8, 0.1 M NaCl, 2 mM MgCl₂, 5 mM dithiothreitol, 0.01% (w/v) Triton X-100.

**Preparation of Tissue Extracts for Assays of Calmodulin N-Methyltransferase, Calmodulin, and Calmodulin N-Methyltransferase Substrate—**Male rats were asphyxiated with CO₂, and tissues were removed and chilled on ice. Tissues were homogenized in 5 volumes of 25 mM Hepes-NaOH, pH 7.4, 0.25 mM sucrose, and 0.01% (w/v) Triton X-100. Supernatants were brought to 35% saturation with solid ammonium sulfate added slowly with constant stirring, stirred an additional 45 min, and centrifuged for 30 min at 15,000 × g. The resulting supernatant was brought to 70% saturation with ammonium sulfate, stirred for 45 min, and centrifuged for 30 min at 15,000 × g. The resulting supernatant was brought to 85% saturation with ammonium sulfate, and was brought to 100% saturation by the addition of solid ammonium sulfate. Supernatants were collected by centrifugation, and quickly frozen at −20 °C. Calmodulin N-methyltransferase assays were performed within 2 weeks of the date of sample preparation.

**Calmodulin Purification—**Frozen packed *D. discoideum* cells were thawed and homogenized in ice-cold 150 mM trichloroacetic acid (18), and precipitated proteins were collected by centrifugation at 30,000 × g for 20 min. The pellet was extracted by homogenization in 50 mM Tris-Cl, pH 7.4, 0.1 mM EGTA, and the extract was clarified by centrifugation at 30,000 × g for 20 min. The extract was mixed with a slurry of DEAE-cellulose (Whatman DE-53) equilibrated in this buffer. The DEAE-cellulose was washed on a Buchner funnel with the equilibration buffer, then with this buffer containing 0.15 M NaCl; calmodulin was then eluted with buffer containing 0.5 M NaCl. This eluate was made 2 M in CaCl₂, clarified by centrifugation, and applied to a column of phenyl-Sepharose (Pharmacia) (19) equilibrated in 50 mM Tris-Cl, pH 7.4, 0.5 M CaCl₂, 0.5 M NaCl. The column was washed with equilibration buffer, and calmodulin was then eluted with 50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 2 mM EGTA. The UV absorbance of the eluate was continuously monitored at 280 nm; eluted protein was concentrated by ultrafiltration and applied to a column of Sephadex G-100 developed with 25 mM NH₄HCO₃. Calmodulin was detected by its absorbance at 280 nm; active fractions were pooled and lyophilized. All procedures were carried out at 4 °C.

**Enzymatic Assay of Calmodulin—**Calmodulin was assayed in boiled tissue extracts by its ability to activate partially purified bovine brain cyclic nucleotide phosphodiesterase. Calmodulin N-methyltransferase assays used here have been described previously (5, 6) and validated (6). Assays were done under conditions where enzyme activity is constant with time and proportional to enzyme concentration. Briefly, reactions were carried out in a volume of 100 µl, using as substrates [methyl-3H]AdoMet (10 µM) and purified Dicyostelium calmodulin (8 µg/tube for the assay of cyclic nucleotide N-methyltransferase activity, or 0.05 µg/tube for the assay of partially purified calmodulin N-methyltransferase activity) and 0.5 mM CaCl₂, 1 mM cyclic AMP, 0.1% (w/v) Lubrol FX, 0.4 mM EGTA, 0.4 mM NaCl for 30 min at 37 °C. Tubes were immersed in boiling water for 2 min, then cooled on ice. Crotalus atrox venom (0.1 ml of a solution containing 0.5 mg/ml) was added and incubated for 30 min at 37 °C. Ice-cold trichloroacetic acid (0.2 ml, 20% w/v) was added, and tubes were centrifuged for 20 min at 2000 × g. Aliquots of 1 ml were removed for the assay of inorganic phosphate (22). The calmodulin concentration in tissue extracts was calculated from the amount of extract required for half-maximal activation of phosphodiesterase; standard curves were made using purified bovine testis calmodulin.

**Calmodulin N-Methyltransferase Assays—**The radiometric calmodulin N-methyltransferase assays used here have been described previously (5, 6) and validated (6). Assays were done under conditions where enzyme activity is constant with time and proportional to enzyme concentration. Briefly, reactions were carried out in a volume of 100 µl, using as substrates [methyl-3H]AdoMet (10 µM) and purified Dicyostelium calmodulin (8 µg/tube for the assay of cyclic nucleotide N-methyltransferase activity, or 0.05 µg/tube for the assay of partially purified calmodulin N-methyltransferase activity) and 0.5 mM CaCl₂, 1 mM cyclic AMP, 0.1% (w/v) Lubrol FX, 0.4 mM EGTA, 0.4 mM NaCl for 30 min at 37 °C. Ice-cold trichloroacetic acid (0.2 ml, 20% w/v) was added, and tubes were centrifuged for 20 min at 2000 × g. Aliquots of 1 ml were removed for the assay of inorganic phosphate (22). The calmodulin concentration in tissue extracts was calculated from the amount of extract required for half-maximal activation of phosphodiesterase; standard curves were made using purified bovine testis calmodulin.

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AdoMet, Sadenosyl-L-methionine; EGTA, ethylenbis(oxyethylenenitriilo)tetracetic acid; HPLC, high pressure liquid chromatography.
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50-fold purified calmodulin N-methyltransferase activity (purified through the CM-Sepharose step, see above) for 5 min at 37 °C in a total volume of 110 μl. Reactions were stopped by the addition of a terminator buffer, modified from Ref. 6, containing 50 mM Tris-HCl, pH 8, 0.3 M NaCl, 0.5 mM MgCl2, 20 μg/ml bovine testis calmodulin, 60 μg/ml bovine serum albumin. Blank was the tissue extracts incubated without purified calmodulin N-methyltransferase. All substrate activities were expressed as a percentage of the value obtained with 1 μg of purified Dicytostelium calmodulin.

Electrophoretic Assay for Calmodulin N-Methyltransferase Substrate Specificity—Tissue cytosolic fractions were prepared as above and incubated with 5 μCi of [methyl-3H]S-adenosylmethionine. All samples were electrophoresed in a discontinuous sodium dodecyl sulfate system by the method of O’Farrell (22). Stacking gels contained 4.5% acrylamide, and resolving gels were 12.5% acrylamide. After electrophoresis, the gel was stained with Coomassie Blue R-250, gels were treated with sodium salicylate for fluorography by the method of Chamberlain (23) and exposed to Kodak XAR-5 film at -70 °C. Molecular weight calibration was done using the following standards: bovine serum albumin, M, 66,000; ovalbumin, M, 45,000; tryptophan synthetase, M, 24,000; β-lactoglobulin, M, 18,000; and lysozyme, M, 14,000.

In Vitro Methylation of Calmodulin—For each 100 μg (6 nmol) of nonmethylated calmodulin, 800 pmol/min partially purified calmodulin N-methyltransferase activity was used. Calmodulin N-methyltransferase, calmodulin, and AdoMet (375 nmol) were incubated for 4 h at 37 °C in closed tubes in a total volume of 1.2 ml containing (final concentrations): 0.1 M glycylglycine-NaOH, pH 8, 0.1 M NaCl, 5 mM dithiothreitol, 2 mM MgCl2, and 0.01% Triton X-100. In some experiments, AdoMet was [3H]-labeled in the methyl moiety (0.05 Ci/mmol). After incubation, the tubes were sonicated at 4 °C for 10 min and then with 3 ml of 0.1 mM CaCl2. Calmodulin was eluted with 3 ml of 25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5 mM CaCl2, and applied to a phenyl-Sepharose column (2-ml bed volume) previously equilibrated with 25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5 mM CaCl2. The column was washed with 8 x 3 ml of equilibration buffer and then with 3 ml of 0.1 mM CaCl2. Calmodulin was eluted with 3 x 2 ml of 2 mM EDTA-NaOH, pH 8. In some experiments this eluate was taken to dryness in vacuo (Speed Vac, Savant Instruments), taken up in 250 μl of water, and injected directly for HPLC. In other experiments the protein was dialyzed against 10 mM NH4HCO3 and lyophilized in aliquots.

HPLC of Calmodulin and Tryptic Peptides—Chromatography was performed on a 70 x 4 mm Ultrapore 3RPSC column (C3-reverse phase, 30-nm pore size, Altex/Beckman). The aqueous component of the mobile phase was 10 mM potassium phosphate, pH 6.1, containing either 2 mM EGTA or 0.2 mM calcium phosphate. The column was equilibrated at a flow rate of 1 ml/min in a mobile phase consisting of the aqueous component mixed with 5% acetonitrile (by volume). At the time of injection, a linear gradient to 37.5% acetonitrile in 40 min was begun; this was followed by a linear gradient to 57% acetonitrile in 5 min. Absorbance of the column effluent was monitored continuously at 280 and 214 nm; the gradient stream was pumped through the reference flow cell of the 214-nm detector to prevent baseline shift. In experiments involving radioactive substrates, a small aliquot of the sample was injected, and fractions were collected for liquid scintillation counting. For preparative runs, peaks were manually collected.

Tryptic Digestion of [3H]Methyl Calmodulin—Calmodulin methylated in vitro with [methyl-3H]AdoMet was resolated by phenyl-Sepharose chromatography and HPLC (using the EGTA-containing buffer). The collected peak from HPLC was dried in a Speed Vac and then redissolved in 0.2 M N-ethylmorpholine/acetate, pH 8.6. Trypsin (1:100, w/w) was added (as 0.1 mg/ml solution in 0.1 mM HCl), the sample was incubated in a covered tube for 4 h at 37 °C, an additional aliquot of trypsin was added (1:100), and the sample was incubated at 37 °C overnight. This solution was filtered and injected directly for HPLC.

Amino Acid Analysis—Samples were taken to dryness in 5 x 30-mm glass tubes and hydrolyzed for 24 h at 110 °C with thioglycollate (2 μl/sample) in 5.6 N HCl in vacuo. Hydrolysates were taken to dryness in a Speed Vac, redissolved in 600 μl of water, rodried, and subjected to amino acid analysis on a Beckman 8200 automated amino acid analyzer. Some hydrolysates were analyzed on a chromatography system designed to resolve lysine, monomethyl-, dimethyl-, and trimethyllysine (6).

Protein Quantiﬁcation—Puriﬁed calmodulin was quantitated by total amino acid analysis. Other protein determinations were done by the method of Bradford (24), using bovine γ-globulin as standard.

Results

Subcellular Distribution of Calmodulin N-Methyltransferase—Studies on the subcellular distribution of calmodulin N-methyltransferase showed that the activity was highest in the cytosolic fraction of rat brain (Table I). Similar results (not shown) were obtained with subcellular fractions of testis, spleen, and kidney. Aliquots of brain fractions were also subjected to freezing/thawing or sonication before assay; only the crude nuclear fraction showed an increase in activity after these treatments, raising the specific activity of this fraction to 15% of the specific activity of the cytosolic fraction at most. All further studies were carried out on cytosolic fractions, which could be assayed by the more convenient phenyl-Sepharose chromatography method.

Tissue Distribution of Calmodulin N-Methyltransferase and Calmodulin—Levels of calmodulin N-methyltransferase activity were determined in seven tissues, and were compared to the calmodulin content of those tissues (Table II). In five of the seven tissues studied, the rank order of calmodulin N-methyltransferase activity was the same as the rank order of calmodulin content. These tissues were from highest calmodulin content to lowest: testis, brain, liver, heart, and skeletal muscle. Spleen and kidney, however, had calmodulin N-methyltransferase levels comparable to brain and testis, but had calmodulin levels similar to liver; calmodulin N-methyltransferase/calmodulin ratios in spleen and kidney were the highest of the tissues studied.

Distribution of Undermethylated Calmodulin—To determine whether low apparent levels of calmodulin N-methyltransferase activity in some tissues were the result of the presence of an inhibitor of this enzyme, we attempted to measure calmodulin N-methyltransferase activities in mixtures of extracts from different tissues. No evidence for an inhibitor was found, but these experiments led to the discovery that tissues with low calmodulin N-methyltransferase levels contain a substrate for calmodulin N-methyltransferase. This substrate is heat stable, and the N-methylated product of the reaction binds to phenyl-Sepharose in a calcium-dependent manner and comigrates with authentic vertebrate calmodulin on electrophoresis (see below). The evidence strongly suggests that the sole calmodulin N-methyltransferase substrate is undermethylated calmodulin, that is an undetermined mixture of calmodulins having lysine, monomethyllysine, or dimethyllysine rather than trimethyllysine at position 115. In several experiments, skeletal muscle, heart, and liver showed an increase in activity after these treatments, raising the specific activity of this fraction to 15% of the specific activity of the cytosolic fraction at most. All further studies were carried out on cytosolic fractions, which could be assayed by the more convenient phenyl-Sepharose chromatography method.

Table I

Subcellular distribution of S-adenosylmethionine:calmodulin (lysine) N-methyltransferase in rat brain

<table>
<thead>
<tr>
<th>Tissue fraction</th>
<th>Fresh</th>
<th>Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pmol min⁻¹ mg protein⁻¹</td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.23 ± 0.14</td>
<td>1.10 ± 0.16</td>
</tr>
<tr>
<td>Nuclear</td>
<td>0.42 ± 0.11</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>0.40 ± 0.07</td>
<td>0.42 ± 0.11</td>
</tr>
<tr>
<td>Microsomal</td>
<td>0.28 ± 0.07</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>4.12 ± 0.17</td>
<td>3.92 ± 0.30</td>
</tr>
</tbody>
</table>
Calmodulin N-Methyltransferase

TABLE II

Tissue distribution of S-adenosylmethionine:calmodulin (lysine) N-methyltransferase (CLNMT), CLNMT substrate, and calmodulin

Samples for CLNMT and calmodulin were prepared from pooled tissues from 4 male rats (300–330 g). CLNMT values represent average of triplicates ± S.D. Samples for CLNMT substrate determinations were prepared from pooled tissues from 4 male rats (280–320 g). Accuracy of calmodulin determinations was checked by adding purified calmodulin to tissue extracts; recovery of added calmodulin was 102 ± 33% (mean ± S.D. of 10 determinations).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CLNMT activity</th>
<th>Total CaM</th>
<th>CLNMT/ CaM</th>
<th>CLNMT substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min⁻¹ mg protein⁻¹</td>
<td>mg g⁻¹, wet weight⁻¹</td>
<td>µg g⁻¹, wet weight⁻¹</td>
<td>pmol min⁻¹ µg⁻¹</td>
</tr>
<tr>
<td>Testis</td>
<td>4.75 ± 0.21</td>
<td>137 ± 6.0</td>
<td>420</td>
<td>0.33</td>
</tr>
<tr>
<td>Brain</td>
<td>4.64 ± 0.26</td>
<td>107 ± 6.1</td>
<td>360</td>
<td>0.30</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.20 ± 0.06</td>
<td>152 ± 4.0</td>
<td>67</td>
<td>2.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.86 ± 0.19</td>
<td>107 ± 11.0</td>
<td>100</td>
<td>1.1</td>
</tr>
<tr>
<td>Liver</td>
<td>0.22 ± 0.02</td>
<td>17 ± 1.5</td>
<td>84</td>
<td>0.20</td>
</tr>
<tr>
<td>Heart</td>
<td>0.15 ± 0.05</td>
<td>6.8 ± 2.3</td>
<td>41</td>
<td>0.17</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.084 ± 0.002</td>
<td>1.7 ± 0.1</td>
<td>38</td>
<td>0.045</td>
</tr>
</tbody>
</table>

and liver (in that order) were found to have the highest levels of calmodulin N-methyltransferase substrate (Table II); and these same tissues had the lowest levels of calmodulin N-methyltransferase and the lowest ratios of calmodulin N-methyltransferase/calmodulin, suggesting that in these tissues calmodulin N-methyltransferase activity is insufficient to keep pace with the rate of calmodulin synthesis.

Purification of Calmodulin N-Methyltransferase—Because the specific activity of calmodulin N-methyltransferase was the highest in cytosolic fraction of testis, this tissue was chosen as the starting material for the purification of calmodulin N-methyltransferase. In early experiments, purification factors of 20-fold were associated with recoveries as low as 5%, regardless of the sequence of purification techniques used. In a subsequent series of experiments, calmodulin N-methyltransferase was partially purified by ammonium sulfate precipitation and DEAE-cellulose chromatography, and a number of compounds were tested for their ability to stabilize the enzyme activity. Sucrose, glyceral, benzamidin, phenylmethylsulfonyl fluoride, and dithiothreitol were ineffective; bovine serum albumin, Triton X-100, Lubrol PX, and polyethylene glycol were all effective in stabilizing calmodulin N-methyltransferase, and their presence resulted in recoveries ranging from 150 to 340% of control (no additions) activity in several experiments. Highest recoveries were obtained in the presence of Triton X-100, and the optimal concentration of this detergent was determined to be 0.01% (w/v); this corresponds to 0.15 mM or about 0.5 times the critical micelle concentration of this detergent (25). The same detergent concentration was optimal regardless of the total protein concentration, and reaction rates were linear with added enzyme under these conditions (data not shown).

When calmodulin N-methyltransferase activity of crude cytosolic fractions was assayed in the presence of 0.01% Triton, enzyme activity was stimulated about 30%, but this stimulatory effect was distinct from the stabilizing effect exerted by Triton at later stages in the purification of this enzyme, in that the calmodulin N-methyltransferase activity of cytosolic fractions remained stable over the course of several hours (or up to 2 weeks, if frozen at -20°C) in the absence of Triton, whereas optimal recoveries during purification required Triton to be present at all times in all purification steps beyond the ammonium sulfate step, as well as in the assay buffers and in buffers used to dilute highly concentrated enzyme in preparation for assay.

Although sulfhydryl-reducing agents alone were ineffective in stabilizing calmodulin N-methyltransferase, the purified enzyme (in the presence of Triton) was stimulated strongly by dithiothreitol; a 50-fold purified enzyme preparation was stimulated 70% by dithiothreitol concentrations of 5 mM and higher; 2-mercaptoethanol was less effective, even at high concentrations. In view of these findings, all column chromatography buffers contained 2 mM dithiothreitol, and the purified enzyme was stored in 10 mM dithiothreitol and assayed in 5 mM dithiothreitol. To make assay conditions consistent, 0.01% Triton X-100 and 5 mM dithiothreitol were added to the standard reaction buffer, regardless of the purification state of the enzyme.

Calmodulin N-methyltransferase does not bind to DEAE-cellulose, but it binds strongly to CM-Sepharose and is eluted at a higher salt concentration than most other proteins (Fig. 1); this suggests that calmodulin N-methyltransferase has numerous basic residues exposed on its surface. Calmodulin N-methyltransferase elutes from Sephadex G-100 with an apparent molecular weight of 150,000 (Fig. 2); we do not know if this behavior would be altered in the absence of Triton X-100. The entire purification procedure developed could be
of calmodulin methylation. Incorporation of [3H]methyl groups into calmodulin was greatest when calmodulin N-methyltransferase was incubated with muscle or liver cytosolic fractions (which have low levels of calmodulin N-methyltransferase), providing further evidence that the calmodulin N-methyltransferase substrate found at high levels in liver and muscle (see above) is undermethylated calmodulin.

Addition of purified calmodulin to tissue extracts resulted in a variable suppression of the methylation of endogenous calmodulin by endogenous calmodulin N-methyltransferase (Fig. 3; also see Refs. 4 and 26) and an enhancement of methyl incorporation into some bands, most strongly a band of 45,000 M<sub>r</sub> in spleen, testis, liver, and kidney extracts. Inhibition of calmodulin methylation under similar conditions (4, 26) as well as calmodulin-stimulated protein methylation (28) have been previously reported by this laboratory. There was inhibition of methyl incorporation into bands at M<sub>r</sub> 40,000 and 45,000 in spleen, testis, liver, and kidney extracts in the presence of added calmodulin N-methyltransferase; the significance of this observation is unknown. The rationale for adding purified calmodulin plus calmodulin N-methyltransferase was that if alternate substrates for calmodulin N-methyltransferase were detected, addition of excess calmodulin should suppress their methylation by competing for available calmodulin N-methyltransferase. In testis cytosolic fraction (Fig. 3B) there was a slight enhancement of methylation of a band at 57,000 M<sub>r</sub> with added calmodulin N-methyltransferase, but this enhancement was not reversed by addition of excess calmodulin. Addition of calmodulin without calmodulin N-methyltransferase also enhanced the methylation of the 57,000 M<sub>r</sub> band, making it unlikely that this band represents an alternate substrate for calmodulin N-methyltransferase.

It was noted that the upper or accessory bands of [3H]methyl calmodulin were seen when the only calmodulin present was the endogenous calmodulin in tissue extracts, as well as when purified calmodulin was added. In another similar experiment (data not shown), cytosolic fractions of brain and spleen were heated for 10 min at 51°C to inactivate endogenous calmodulin N-methyltransferase and then incubated with added calmodulin N-methyltransferase or calmodulin, or both. When heat-treated cytosolic fractions were used, the [3H]-methylated upper band appearance was substantially reduced, both in the case of added calmodulin N-methyltransferase alone and in the case where calmodulin N-methyltransferase and calmodulin were added, suggesting that at least part of the modified calmodulin detected in this experiment was produced during the incubation of the tissue extracts for methylation and that the activity or activities responsible for the modification were inactivated by heat treatment.

When the polyacrylamide gel from the calmodulin N-methyltransferase substrate specificity experiment was subjected to fluorography for 60 days (not shown), a faint band of [3H]methyl incorporation was seen at 38,000 M<sub>r</sub> in the gel lane containing calmodulin N-methyltransferase alone. This methylation was completely suppressed by the addition of calmodulin. No corresponding [3H]-methylated band was seen in testis cytosolic fraction (from which calmodulin N-methyltransferase was purified) when it was incubated either with or without added purified calmodulin N-methyltransferase. A similar faint band was seen on the overexposed fluorogram of a gel electrophoresis experiment that used 48-fold purified calmodulin N-methyltransferase (not shown) in place of the 470-fold purified enzyme used here. One interpretation of this band is that it represents an alternate substrate for calmodulin N-methyltransferase, which copurifies with the enzyme; another interpretation is that it represents calmodulin N-
methyltransferase itself (or a subunit of calmodulin N-methytransferase) and that in the absence of substrate the enzyme itself becomes methylated (or otherwise \(^{3}H\)-labeled, see "Discussion").

Similar studies of in vitro methylation in the presence of added calmodulin N-methyltransferase were carried out using whole tissue homogenates, homogenates prepared in hypotonic buffer, and soluble fractions prepared by centrifugation of tissues homogenized in hypotonic buffer. Results of these experiments were identical to the studies using cytosol, in that calmodulin was the only detectable substrate for calmodulin N-methyltransferase in all tissues examined.

Stoichiometry of in Vitro Methylation of Calmodulin—To determine the maximal extent of in vitro methylation, 30 \(\mu\)g (1.8 nmol) of Dictyostelium calmodulin was incubated with calmodulin N-methyltransferase and \([\text{methyl-}^{3}H]\text{AdoMet}, \) and aliquots of the reaction mixture were withdrawn for assay at various times. The reaction proceeded rapidly beyond 1 mol of methyl groups/mol of calmodulin within 10 min, then gradually slowed, reaching apparent completion at 2 h, with a maximal incorporation of 3 mol of methyl groups/mol of calmodulin (Fig. 4). In several experiments using other batches of Dictyostelium calmodulin, maximal incorporation ranged from 2.7 to 3.3 mol of methyl groups/mol of calmodulin. Reactions which had run to completion could be restarted by the addition of more Dictyostelium calmodulin, indicating that the enzyme remained active (data not shown).

To determine the amino acid residues into which \(^{3}H\) methyl groups were incorporated, in vitro methylation reactions were stopped at various times and subjected to the phenyl-Sepharose assay, and \(^{3}H\)-methylated calmodulin eluted from the phenyl-Sepharose column with EDTA was divided, half for liquid scintillation counting, and the other half hydrolyzed for analysis of methylated basic amino acids. All tritium in the hydrolysates was associated with methyl lysines, as in previous experiments (6). At 6 min (corresponding to 50% completion in this experiment), tritium label was found in mono-, di-, and trimethyllysines; at 2 h (at or near apparent completion), nearly all radioactivity was associated with trimethyllysine, with a trace of monomethyllysine also detectable (Fig. 5). These results are consistent with the conclusion that in vitro methylation of Dictyostelium calmod-
purified calmodulin N-methyltransferase, third residue of version of lysine 115 to trimethyllysine and, therefore, that with its identification as peptide 107-126 of calmodulin; this peptide contains lysine 115 as its only lysine residue (Table IV). It was expected that trypsin should cleave the protein at arginine 106 and arginine 126, but not at trimethyllysine 115, because the homologous peptide with internal trimethyllysine 115 was generated by tryptic digestion of bovine calmodulin (29) and because trimethyllysyl residues are not in general targets for tryptic cleavage (30). From these results we conclude that in vitro methylation of Dictyostelium calmodulin by rat testis calmodulin N-methyltransferase results in conversion of lysine 115 to trimethyllysine and, therefore, that in vitro methylated Dictyostelium calmodulin is appropriate for use in experiments to determine the biological effect of trimethylation of lysine 115 in calmodulin.

Effects of Methylation on Calmodulin Activity—As a control for any possible side effects of 4-h incubation with partially purified calmodulin N-methyltransferase, in vitro methylated calmodulin was compared with Dictyostelium calmodulin incubated with calmodulin N-methyltransferase in the absence of AdoMet (sham methylated). In a preliminary experiment, untreated Dictyostelium calmodulin was compared with in vitro methylated and sham methylated Dictyostelium calmodulins reisolated from methylation reaction mixtures by phenyl-Sepharose chromatography alone. All three proteins comigrated on electrophoresis and exhibited the “EGTA shift” in mobility characteristic of all calmodulins, e-N-trimethyllysine; Melys, t-N-monomethyllysine.
Unlabeled "H-methylated calmodulin and composition found for labeled tryptic peptide (3.4 nmol, as calculated from "H content of an aliquot) from Dictyostelium calmodulin (lysine) N-methyltransferase. [3H]Methyl incorporation was 2.5 mol/mol of calmodulin.

Aliquots of 2 µg were electrophoresed on MINI-SLAB gels (Idea Scientific) with the addition of gel was 12.5% acrylamide, and the resolving gel was 381.

Methylated calmodulin. Partially purified bovine brain cyclic nucleotide phosphodiesterase (20) was assayed as described under "Materials and Methods" except that the reaction buffer contained 25 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 0.5 mM CaCl₂, and 1 mM cyclic AMP. Phosphodiesterase was activated with bovine testis calmodulin (●), sham methylated Dictyostelium calmodulin (○), or methylated Dictyostelium calmodulin (△). Each point represents the average of duplicates.

In vitro methylated and sham methylated calmodulin (re-isolated from reaction mixtures by phenyl-Sepharose chromatography followed by HPLC) comigrated in SDS electrophoresis in the presence of EGTA (Fig. 8) or calcium (data not shown). In the presence of calcium, a second minor component was evident in both proteins to a similar extent. Bovine calmodulin and in vitro methylated and sham methylated Dictyostelium calmodulin were tested for their ability to activate phosphodiesterase from bovine brain; there were no detectable differences between the three calmodulins, either in the amount required for half-maximal activation or in the level of maximal activation (Fig. 9).

**DISCUSSION**

We have presented data on the quantitative subcellular distribution of calmodulin N-methyltransferase and have shown that the majority of the activity is cytosolic (Table I). Since the majority of tissue calmodulin is also cytosolic, its methylation could be either cotranslational or post-translational. This is in contrast to the methylation of histones, which occurs in the nucleus after the histones have been incorporated into chromatin (13, 31).

Tissue distribution studies of calmodulin N-methyltransferase were undertaken for two reasons. First, it was hoped that a comparison of the distribution of the enzyme and calmodulin would indicate whether calmodulin was the only substrate for calmodulin N-methyltransferase or whether we might expect to find other substrates. Second, we wanted to learn the best source of calmodulin N-methyltransferase for purification of this enzyme. Results of the distribution study were equivocal with respect to the question of the specificity of calmodulin N-methyltransferase; the agreement of rank order of calmodulin and calmodulin N-methyltransferase in testis, brain, liver, heart, and skeletal muscle was strongly suggestive of specificity. However, there was a 50-fold range of calmodulin N-methyltransferase/calmodulin ratios among the 7 tissues studied (Table II).

High ratios of calmodulin N-methyltransferase/calmodulin in some tissues suggested that there might be substrates other than calmodulin for calmodulin N-methyltransferase in these tissues, but we did not detect any other substrates (Fig. 3). This extends the findings of Sitaramayya et al. (4), who found that cytaybrome c and histones, which are known to be

**TABLE IV**

Amino acid composition of [3H]methyl peptide

Predicted composition of peptide 107–126 of Dictyostelium calmodulin and composition found for labeled tryptic peptide (3.4 nmol, as calculated from "H content of an aliquot) from Dictyostelium calmodulin (lysine) N-methyltransferase. [3H]Methyl incorporation was 2.5 mol/mol of calmodulin.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Predicted</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>2</td>
<td>1.82</td>
</tr>
<tr>
<td>Thr</td>
<td>2</td>
<td>1.91</td>
</tr>
<tr>
<td>Ser</td>
<td>1</td>
<td>0.96</td>
</tr>
<tr>
<td>Glu</td>
<td>4</td>
<td>2.92</td>
</tr>
<tr>
<td>Pro</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gly</td>
<td>1</td>
<td>1.09</td>
</tr>
<tr>
<td>Ala</td>
<td>0</td>
<td>0.18</td>
</tr>
<tr>
<td>Val</td>
<td>2</td>
<td>1.31</td>
</tr>
<tr>
<td>Cys</td>
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<td>0</td>
</tr>
<tr>
<td>Met</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>Leu</td>
<td>2</td>
<td>1.92</td>
</tr>
<tr>
<td>Tyr</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Me3Lys</td>
<td>c</td>
<td>0.26</td>
</tr>
<tr>
<td>Lys + Me3Lys</td>
<td>c</td>
<td>0.82</td>
</tr>
<tr>
<td>Melys</td>
<td>c</td>
<td>0</td>
</tr>
<tr>
<td>His</td>
<td>1</td>
<td>0.69</td>
</tr>
<tr>
<td>Arg</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td>Trp</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Methionine was evidently lost during trypsin treatment or HCl hydrolysis.
  * Me3Lys, ε-N-trimethyllysine; Me3Lys, ε-N-dimethyllysine; Melys, ε-N-monomethyllysine.
  * The sum of lysine plus mono-, di-, and trimethyllysine was expected to be 1.
  * ND, not determined.

**FIG. 8.** Electrophoresis of methylated calmodulin. Methylated and sham methylated Dictyostelium calmodulin were reisolated from reaction mixtures by phenyl-Sepharose chromatography and HPLC in the presence of calcium (see "Materials and Methods"). Aliquots of 2 µg were electrophoresed on MINI-SLAB gels (Idea Scientific) 6 cm long, 0.8 mm thick. Electrophoresis was performed as described by O'Farrell (22), with the addition of 4 mM EGTA to each sample (27). The stacking gel was 4.5% acrylamide, the resolving gel was 12.5% acrylamide, and the acrylamide/bisacrylamide ratio was 38:1.

**FIG. 9.** Phosphodiesterase activation by *in vitro* methylated calmodulin. Partially purified bovine brain cyclic nucleotide phosphodiesterase (20) was assayed as described under "Materials and Methods" except that the reaction buffer contained 25 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 0.5 mM CaCl₂, and 1 mM cyclic AMP. Phosphodiesterase was activated with bovine testis calmodulin (●), sham methylated Dictyostelium calmodulin (○), or methylated Dictyostelium calmodulin (△). Each point represents the average of duplicates.
Calmodulin N-Methyltransferase

substrates for other protein (lysine) N-methyltransferases, are not substrates for calmodulin (lysine) N-methyltransferase. We cannot rule out the possibility that other substrates exist and that they were not detected with the electrophoresis and fluorography assay used.

In tissues with low calmodulin N-methyltransferase/calmodulin ratios, calmodulin is incompletely methylated (Table II). Our finding indicating different methylation states for calmodulins from different organs of the same organism may explain conflicting results obtained by other investigators in studies on calmodulin from octopus; calmodulin from the optic lobe of this animal was found to contain 0.6 mol of trimethyllysine/mole of protein (32), while calmodulin isolated from whole octopus had 0.1 mol each of mono-, di-, and trimethyllysine/mole of protein (33).

Our finding of variable methylation states for calmodulin may also explain an apparent contradiction between quantitative in vitro calmodulin N-methyltransferase assays and data from electrophoresis-fluorography experiments. Although in vitro assays indicate great variation in the tissue content of calmodulin N-methyltransferase (Table II, see also Ref. 6), electrophoresis experiments indicate a relatively constant level of incorporation of methyl groups into endogenous calmodulin by endogenous calmodulin N-methyltransferase in extracts of several tissues (compare left lanes of skeletal muscle, spleen, and testes in Fig. 2B, also see Refs. 6, 26, and 28). In those experiments where no exogenous calmodulin was added, methyl incorporation in a given tissue is a function of the activity of calmodulin N-methyltransferase and of the concentration and degree of methylation of calmodulin in that tissue. Skeletal muscle contains little calmodulin N-methyltransferase and little calmodulin, but the calmodulin that is present is an excellent substrate for further methylation. Testes and spleen contain much more calmodulin N-methyltransferase, but the calmodulin in these tissues is a poor substrate for further methylation (Table II). There is an approximately reciprocal relationship between calmodulin N-methyltransferase and calmodulin N-methyltransferase substrate levels in various tissues, resulting in a relatively constant incorporation of methyl groups seen in electrophoresis experiments.

Evidence has been presented here that rat testis calmodulin N-methyltransferase can be used to methylate Dictyostelium calmodulin in vitro to the extent of 1 mol of trimethyllysine/mole of calmodulin. In vitro methyltransferase occurs at position 115, the same lysine residue which is methylated in vivo in calmodulins of most species (1). Conditions established for the in vitro preparative methylation of calmodulin include a relatively low ratio of calmodulin to calmodulin N-methyltransferase and a relatively long incubation time. Although it is not surprising that close to 100% of the calmodulin substrate can be trimethylated under these conditions, our results stand in striking contrast to the results obtained in studies on the in vitro methylation of cytochrome c by enzymes derived from several sources (9–11). In those studies, it was necessary to use a relatively large amount of cytochrome c unit of methyltransferase ($K_v$ values for native horse heart cytochrome c ranged from 1 to 6 mM), and incorporation of methyl groups ceased after methylation of only a small fraction of the cytochrome c, suggesting that the methylating enzyme had been inactivated under conditions of the reaction. The reaction, once it had stopped, could be restarted by the addition of more enzyme (9). The appearance of trimethyllysine in the product in spite of a great excess of unreacted substrate suggests that cytochrome c N-methyltransferase does not dissociate from its substrate between the transfer of the first and second or second and third methyl groups (9). Calmodulin N-methyltransferase, in contrast, is stable under conditions of the reaction, as indicated by the restarting of the reaction by the addition of more calmodulin substrate (not shown). The appearance of large amounts of mono- and dimethyllysine early in the course of the reaction (Fig. 5) also suggests that calmodulin dissociates from calmodulin N-methyltransferase between successive methylations; proof of this would involve an investigation of the effect of changes in the enzyme/substrate ratio.

When purified calmodulin N-methyltransferase was incubated with [methyl-$^3$H]AdoMet and the reaction products were analyzed by electrophoresis and fluorography, a faint band of radioactivity was seen at 38,000 $M_r$, in 60-day exposures of the fluorogram (not shown). Three methyltransferases, phenylethanolamine N-methyltransferase (34, 35), catechol O-methyltransferase (34), and protein carboxylmethyltransferase (35), have been shown to undergo photoactivated covalent labeling by [methyl-$^3$H]AdoMet. In these cases, the presence of a methyl acceptor substrate blocked the labeling. In our experiment, addition of calmodulin blocked tritium incorporation into the 38,000 $M_r$ band. Thus, it is possible that the 38,000 $M_r$ band represents calmodulin N-methyltransferase or a subunit of calmodulin N-methyltransferase.

Our finding that N-methylation of calmodulin has no effect on the activation of phosphodiesterase in vitro (Fig. 9) suggests that methylation of calmodulin has no direct or indirect effect on the activation of phosphodiesterase in vivo. This is in contrast to the effects on calmodulin activation of NAD kinase, which is inhibited when des(methyl)calmodulin is methylated by calmodulin N-methyltransferase, as reported in a separate communication (36).

The lack of effect on phosphodiesterase activation is also of technical interest, since we have used phosphodiesterase activation to quantitate calmodulin in tissue extracts, and we have also found that calmodulin from muscle, heart, and liver is less than fully methylated (Table II). Results presented here suggest that undermethylation of calmodulin will have no effect on the quantitation of tissue calmodulin by phosphodiesterase in vitro.

Trimethylation of lysine blocks the action of trypsin at lysine residues (30), and one hypothesis about lysine N-methylation is that it protects proteins from proteolysis (37). This hypothesis was addressed in studies of the effect of N-methylation on the stability of cytochrome c, and the conclusion reached was that the stabilization conferred on cytochrome c by methylation was solely a result of enhanced binding of the protein to mitochondrial membranes (37). A recent study, however, revives the hypothesis that N-methylation protects proteins from proteolysis; it has been reported that Dictyostelium calmodulin is a substrate for degradation by the ATP/ubiquitin-dependent proteolytic system of reticulocytes and that degradation of Dictyostelium calmodulin by this system involves attachment of ubiquitin by an isopeptide linkage to the ε-amino group of lysine 115 of Dictyostelium calmodulin (38). Vertebrate calmodulin was found not to be a substrate for conjugation or degradation, the implication being that N-methylation of lysine 115 protects vertebrate calmodulin from the ATP/ubiquitin-dependent proteolytic system (38). This system has been studied primarily in reticulocytes (39), but its presence has been demonstrated in other tissues (40), and ubiquitin has been found in most eucaryotic tissues (41), suggesting that this proteolytic system may be as widespread as calmodulin. It would be of interest to know the characteristics of the ATP/ubiquitin-dependent proteolytic system in tissues and organisms with nonmethylated or un-
dermethylated calmodulin. Perhaps in such tissues and orga-

nisms the ATP/ubiquitin-dependent proteolytic system is less
active or has different substrate specificity.

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