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Isolation and characterization of calmodulin from spinach leaves and *in vitro* translation mixtures

(trimethyllysine/phenothiazine-Sepharose/calcium-modulated proteins/electrophoretic mobility)

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ABSTRACT **Calmodulin, a multifunctional calcium-modulated protein, has been isolated from spinach leaf tissue and from spinach leaf messenger RNA translation products. The translation protein and the spinach leaf protein have been partially characterized and compared to vertebrate calmodulins. Spinach leaf calmodulin will quantitatively activate bovine brain phosphodiesterase and will undergo a calcium-dependent shift in electrophoretic mobility similar to that of bovine brain calmodulin. In the presence of Ca^{2+} the spinach and brain proteins comigrate, but in the presence of chelators they do not. A polyadenylylated RNA fraction has been isolated from spinach leaf tissue and translated in a wheat germ cell-free translation system. The calmodulin synthesized *in vitro* has been isolated by using calcium-dependent affinity chromatography on phenothiazine-Sepharose conjugates. The translation protein comigrates with spinach calmodulin during polyacrylamide gel electrophoresis whether in the presence or the absence of Ca^{2+}. The translation protein also undergoes a calcium-dependent mobility shift identical to that of spinach calmodulin. Amino acid analysis of the translation calmodulin indicates that it does not contain N^{ϵ}-trimethyllysine, an amino acid residue that is characteristic of all calmodulins previously examined. These studies suggest that N^{ϵ}-trimethyllysine is not required for the calcium-dependent interaction of calmodulin with phenothiazines and indicate the potential utility of phenothiazine-Sepharose conjugates as affinity-based adsorbents in biological and biochemical investigations.**

Calmodulin is a calcium-binding protein with no known enzymatic activity. However, calmodulin will stimulate the activity of several enzymes, affect *in vitro* tubulin polymerization, and bind a class of antipsychotic drugs termed phenothiazines (for a review see ref. 1). The complete amino acid sequence of bovine brain calmodulin has been determined (2, 3) and the physical, chemical, and functional properties of calmodulins isolated from a variety of vertebrate, invertebrate, and plant sources have been compared to those of the well-defined bovine brain protein (3–8). Characteristics of calmodulins examined to date include the presence of a single residue of N^{ϵ}-trimethyllysine, a single residue of histidine, and the lack of tryptophan and phosphate (1–11). The histidyl and trimethyllysyl residues are located, respectively, at sequence positions 107 and 115 in the bovine brain protein (3). Comparative sequence analyses of bovine uterus (12), rat testis (11), and *Renilla reniformis* (4) calmodulins have demonstrated a conservation of the relative sequence positions of these two residues. Three-dimensional models (1, 3, 13) of the calcium-modulated protein family place the histidyl and trimethyllysyl residues between two calcium-binding domains.

The exact functional role of the trimethyllysine in calmodulin is not known. Nuclear magnetic resonance studies indicate a change in the environment of the trimethyllysyl residue upon binding calcium (14). Studies of calmodulin and troponin C as inhibitors of kinase-mediated phosphorylation of troponin I indicate that a region of calmodulin that includes the histidine and trimethyllysine interacts specifically with troponin I (15). These data suggest that the portion of calmodulin containing the trimethyllysine may be involved in calcium-dependent interactions with other macromolecules. In addition, no detectable trypsin cleavage occurs at the trimethyllysyl residue (2, 3) suggesting a possible role for trimethyllysine in the turnover of calmodulin.

The mechanism of calmodulin trimethylation and whether it is a cotranslational or posttranslational event is not known. It is also not known whether calmodulin is made *in vitro* or *in vivo* as a precursor. In order to study the synthesis of calmodulin, to elucidate the mechanism of the trimethylation reaction, and to study the role of trimethyllysine in the turnover and functions of calmodulin, we have isolated and characterized calmodulin synthesized in a wheat germ cell-free translation system. This polypeptide appears to lack trimethyllysine, interacts in a calcium-dependent manner with phenothiazine-Sepharose conjugates, undergoes calcium-dependent mobility shifts during polyacrylamide gel electrophoresis, and comigrates during polyacrylamide gel electrophoresis with mature methylated calmodulin from the same tissue and species.

MATERIALS AND METHODS

Calmodulin was isolated from spinach leaves (*Spinacea oleracea*) by three different procedures, which resulted in the isolation of homogeneous products that had identical physical, chemical and functional properties. Details of the isolation and further characterization of plant leaf calmodulin will be presented elsewhere;* the isolation of spinach leaf calmodulin and of calmodulin from cell-free translation mixtures by using chromatography on 2-chloro-10-(3-aminopropyl)phenothiazine-Sepharose conjugates (CAPP-Sepharose) is summarized below. Calmodulin was isolated from bovine brain and chicken gizzard by using previously described procedures (8, 9) and by using CAPP-Sepharose chromatography (16).

CAPP-Sepharose conjugates were prepared and used essentially as described by Jamieson and Vanaman (16). The 2-chloro-10-(3-aminopropyl)phenothiazine hydrochloride, a gift from Albert Manian of the National Institutes of Health, was dissolved prior to coupling in 50% (vol/vol) ethanol that had been acidified with Ultrex hydrochloric acid. The solution was

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Abbreviations: CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

* D. M. Watterson, D. B. Iverson, and L. J. Van Eldik, unpublished.

added slowly at room temperature to cyanogen bromide-activated Sepharose purchased from Pharmacia and prepared according to the manufacturer's suggestions. Chromatography of samples on CAPP-Sepharose was done in buffer F (10 mM Tris·HCl/1 mM $MgCl_2$/1 mM 2-mercaptoethanol/2 mM $CaCl_2$, pH 8.0) or buffer E (10 mM Tris·HCl/1 mM $MgCl_2$/1 mM 2-mercaptoethanol/2 mM EGTA, pH 8.0) (EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid). Samples were applied to a column (1 × 15 cm) of CAPP-Sepharose equilibrated with buffer F, and the column was washed with three column volumes of buffer F followed by five column volumes of buffer F containing 0.2 M NaCl. Calmodulin was step eluted with buffer E containing 0.2 M NaCl and the column was washed subsequently with buffer E containing 8.0 M urea.

Spinach leaves, obtained fresh from a local vendor, were minced, then homogenized in either buffer I (100 mM Tris·HCl/2 mM EGTA/2 mM 2-mercaptoethanol, pH 8.0) or buffer H (50 mM Tris·HCl/1 mM EGTA/1 mM 2-mercaptoethanol, pH 8.0). When buffer I was used the resultant homogenate was filtered through cheesecloth and centrifuged at 10,000 × *g* for 30 min, and the supernatant was adjusted to 50% (vol/vol) ethanol. The ethanol mixture was centrifuged at 10,000 × *g* for 30 min, and the supernatant was adjusted to 80% (vol/vol) ethanol and centrifuged at 10,000 × *g* for 1 hr. The final pellet was either subjected directly to CAPP-Sepharose chromatography or subjected to chromatography on DEAE-Sephadex A-50 followed by CAPP-Sepharose chromatography. When buffer H was used for homogenization a pH 4.1 pellet was prepared as described (8), resuspended in water, adjusted to pH 7.5, and dialyzed against buffer F. After dialysis the sample was centrifuged at 100,000 × *g* for 60 min and the supernatant was subjected to chromatography on CAPP-Sepharose.

For poly(A)$^+$ RNA isolation, leaves of hydroponically grown spinach were obtained from G. Lorimer (Du Pont), petioles were removed, and high molecular weight RNA was isolated according to the method of Cashmore *et al.* (17). The RNA was precipitated with 2 M lithium chloride then precipitated twice with 2 vol of ethanol. Poly(A)$^+$ RNA was obtained by chromatography on poly(U)-Sepharose 4B conjugates and stored at −80°C in sterile distilled water. The poly(A)$^+$ RNA was translated in a wheat germ system as described (18), except that ^{35}S-labeled methionine (>600 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) at 75 μCi/ml, 0.5 μM methionine, and 1 mCi of ^{3}H-labeled lysine (75 Ci/mmol, lyophilized and resuspended in 1 mM Hepes/KOH, pH 7.5) per ml were included. Oxaloacetate (24 μM) and citrate synthase (100 units) were also added. Translation mixtures were incubated at 26°C for 90 min.

Calmodulin was isolated from translation mixtures by using the buffer I procedure described above. After translation, the mixture was centrifuged at 140,000 × *g* for 1 hr. The supernatant was mixed with 2 vol of buffer I, adjusted to 50% (vol/vol) ethanol, processed through the 80% (vol/vol) ethanol treatment, and chromatographed on CAPP-Sepharose as described for spinach leaf calmodulin.

Samples for amino acid analysis were hydrolyzed and composition analyses were done as described (8). N^ϵ-Trimethyllysine determinations were done by using an isothermal program (80°C) and buffers of pH 3.25, 0.20 M Na^+ (buffer A); pH 4.25, 0.20 M Na^+ (buffer B); and pH 5.00, 0.20 M Na^+ (buffer C). Samples were injected onto the column equilibrated with buffer A. Elution with buffer B was started 6 min after injection and elution with buffer C started 16 min after injection. The elution times of several amino acid standards were: lysine, 78.5 min; N^ϵ-monomethyllysine, 82.8 min; N^ϵ-dimethyllysine, 75 min; N^ϵ-trimethyllysine, 68.1 min; histidine, 80.8 min; 1-methylhistidine, 83.0 min; 3-methylhistidine, 80.6 min; methionine, 23.0 min. The presence of methylated lysyl residues was also determined by using a temperature gradient program. Buffers were the same as those used in the isothermal program and a temperature change from 80°C to 30°C was begun 20 min after sample injection. Under these conditions the elution times of the amino acid standards were: lysine, 89.5 min; N^ϵ-monomethyllysine, 94.6 min; N^ϵ-dimethyllysine, 93.8 min; N^ϵ-trimethyllysine, 89.0 min; histidine, 124.3 min; 1-methylhistidine, 117.3 min; 3-methylhistidine, 139.0 min; methionine, 23.0 min.

Fluorography was done according to the procedure of Bonner and Laskey (19). Polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate was performed essentially as described (8); details are given in the appropriate figure legends. Phosphodiesterase was prepared and assayed as described elsewhere (20). Isotopic samples were quantified by using Aquasol-2 (New England Nuclear) scintillant and a Beckman LS8000 liquid scintillation counter. Distilled deionized water was from a Darco water system (Durham, NC). Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Atomergic Chemetals (formerly Gallard-Schlesinger). Molecular weight standards for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were purchased from Sigma and Boehringer-Mannheim. Ultrapure urea was obtained from Schwarz/Mann. N^ϵ-Trimethyl-L-lysine bis(*p*-hydroxyazobenzene-*p*-sulfonate) was purchased from Calbiochem and converted to N^ϵ-trimethyl-L-lysine by passage through a column of Bio-Rad AG-1-X8 resin (200–400 mesh, acetate form). Isotopes were obtained from New England Nuclear. Wheat germ was a gift from General Mills. All other chemicals were of the highest purity reagent-grade commercially available and used without further purification.

RESULTS

A small acidic protein was isolated from spinach leaf tissue by using the calcium-dependent phenothiazine-Sepharose purification protocol summarized under *Materials and Methods*. This spinach leaf protein possessed a number of characteristics similar to those of vertebrate calmodulins. As evidenced by its isolation by using affinity methods developed for the purification of vertebrate calmodulins (16, 21), spinach leaf calmodulin was also able to bind phenothiazine-Sepharose conjugates in a calcium-dependent manner. Spinach calmodulin quantitatively activated bovine brain phosphodiesterase in a manner indistinguishable from that of the bovine brain calmodulin and to the same extent (Fig. 1). It had a gel electrophoretic mobility similar to vertebrate calmodulin in both the presence and the absence of sodium dodecyl sulfate and showed a calcium-dependent mobility shift in the presence of sodium dodecyl sulfate (Fig. 2). Furthermore, this protein has an amino acid composition similar to that of bovine brain calmodulin, including the presence of N^ϵ-trimethyllysine, and quantitatively competes with bovine brain calmodulin in a radioimmunoassay using affinity-purified antiserum against vertebrate calmodulin.*

One readily detectable difference between plant and vertebrate calmodulins that is germane to the characterization of cell-free translation products is the degree of mobility shift when calmodulins are analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the presence of either Ca^{2+} or EGTA. Burgess *et al.*† have shown that the mobility of calmodulin during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate will vary as a function of

† Burgess, W. H., Jemiolo, D. K. & Kretsinger, R. (1980) *Biochim. Biophys. Acta*, in press.

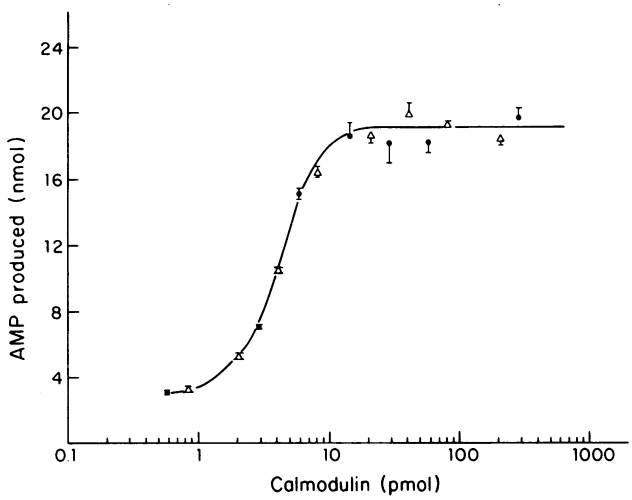

FIG. 1. Phosphodiesterase-stimulatory activity of calmodulin. The quantitative ability of bovine brain calmodulin (●) and spinach calmodulin (▲) to stimulate "activator-depleted" bovine brain 3',5'-cyclic nucleotide phosphodiesterase was determined. A reaction volume of 250 μl containing 2 mM cyclic AMP was used in the experiment shown. Reaction mixtures were analyzed for AMP production and cyclic AMP degradation. Points represent the mean ± SD of triplicate determinations.

the protein-bound calcium; the more calcium bound the greater the mobility. Other proteins in the same family of Ca^{2+}-modulated proteins, such as troponin C, parvalbumin, and S-100b, do not undergo this large mobility shift. When spinach calmodulin was analyzed by polyacrylamide gel electrophoresis in the presence of calcium, or with no additions, it comigrated with, or similarly to, vertebrate calmodulins (Fig. 2 *A–C*). When analysis was done in the presence of EGTA, a large shift in mobility was seen with vertebrate and plant calmodulin (Fig. 2*D*). However, the plant calmodulin did not undergo as large a shift in mobility as did the vertebrate calmodulins. An experiment in which spinach and bovine brain calmodulin were mixed before sample preparation and analysis showed the same mobility difference (Fig. 2*D*).

The molecular mass of bovine brain calmodulin calculated from its amino acid sequence is 16,680 daltons (3) and the molecular weight from sedimentation equilibrium studies done in the presence of 6 M guanidine hydrochloride is 15,256 (9). The estimated molecular weight from analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and EGTA was 18,000–20,500 (Fig. 2*D*). The estimated molecular weight of spinach calmodulin from analysis by polyacrylamide gel electrophoresis under identical conditions was 17,000–19,000 (Fig. 2*D*). A value of 18,000 was obtained for the spinach calmodulin when analyses were done with neither Ca^{2+} nor EGTA added to the sodium dodecyl sulfate buffer, gel, or sample (Fig. 2*A*). The estimated molecular weight of spinach calmodulin in the presence of calcium and sodium dodecyl sulfate was 13,500–15,000 (Fig. 2*C*). It is not known from these studies whether spinach calmodulin actually differs from vertebrate calmodulins in the length of the polypeptide chain or whether it has different calcium-binding properties.

A poly(A)$^+$ mRNA fraction isolated from spinach leaf tissue was translated in a cell-free wheat germ system as described under *Materials and Methods*. When translation mixtures were subjected to phenothiazine-Sepharose chromatography under conditions identical to those used to isolate calmodulins from spinach leaf and other tissue sources, a peak of radioactivity was step-eluted with EGTA (Fig. 3). This material was analyzed by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate and in the presence of sodium dodecyl sulfate containing either Ca^{2+} or EGTA. Under all conditions a single major band of radioactivity that comigrated with spinach calmodulin was observed (Fig. 2). The translated polypeptide also comigrated with spinach calmodulin when mixed and analyzed in the same sample well. Further, the material from the translation mixture underwent a calcium-

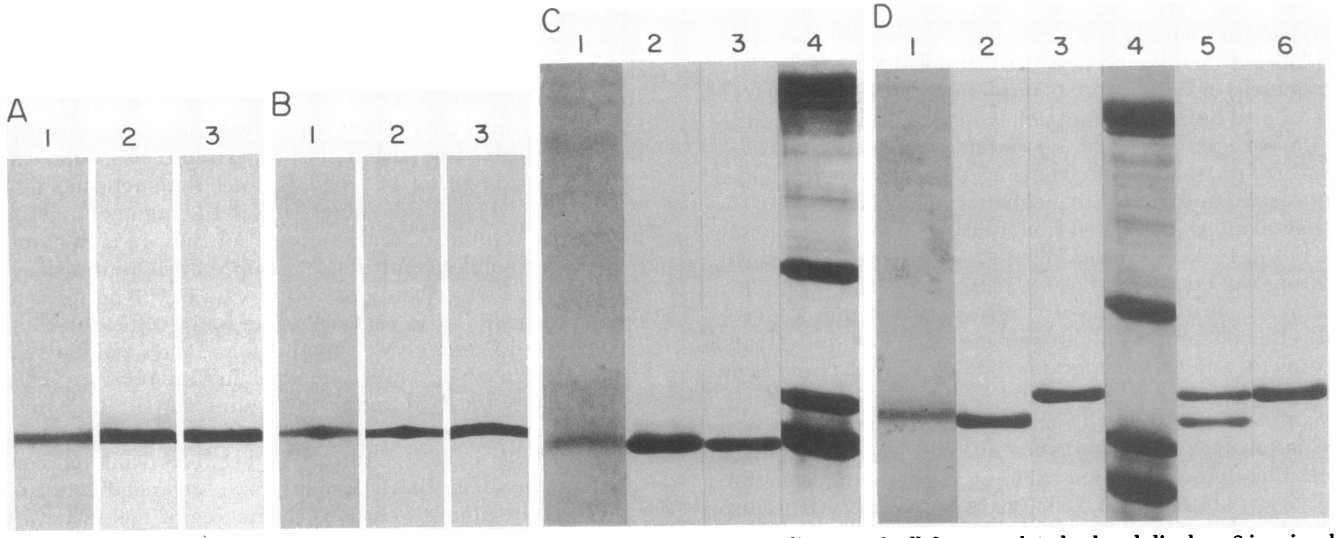

FIG. 2. Electrophoretic analysis of calmodulins. In all panels, lane 1 is an autoradiogram of cell-free translated calmodulin, lane 2 is spinach leaf calmodulin (10 μg), and lane 3 is chicken gizzard calmodulin (10 μg). Lanes 2–6 were stained with Coomassie blue. (*A*) Electrophoresis in the presence of sodium dodecyl sulfate and utilizing a gradient of 8–18% (wt/vol) acrylamide. Samples were incubated at 100°C for 3 min before analysis. (*B*) Electrophoresis in the absence of sodium dodecyl sulfate on a 12.5% (wt/vol) acrylamide gel. (*C*) Electrophoresis in the presence of sodium dodecyl sulfate on a 12.5% (wt/vol) acrylamide gel containing 1 mM Ca^{2+}. All samples and gel buffer also contained 1 mM Ca^{2+}. Samples were incubated at 100°C for 3 min before analysis. Lane 4 contained 10 μg of each molecular weight standard. From top to bottom, the standards are bovine serum albumin (68,000), catalase (60,000), chymotrypsinogen (27,500), myoglobin (17,200), and lysozyme (14,300). (*D*) Electrophoresis as in *C* except that the gel, gel buffer, and samples contained 1 mM EGTA. Lanes 1–4 are as described for C. Lane 5 contained a mixture of spinach leaf calmodulin (5 μg) and bovine brain calmodulin (5 μg). Lane 6 is bovine brain calmodulin (10 μg).

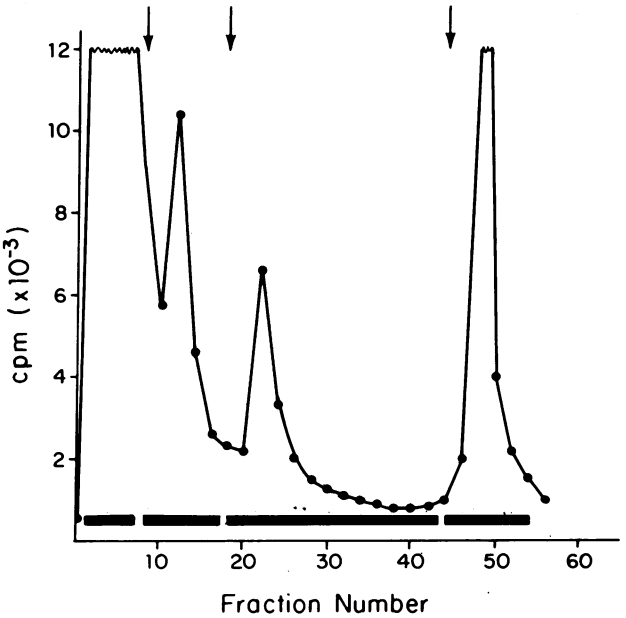

FIG. 3. CAPP-Sepharose chromatography of spinach translation mixture. Samples were applied to the column and washed with buffer F. The arrows represent, from left to right, washes with buffer F + 0.2 M NaCl, buffer E + 0.2 M NaCl, and buffer E + 8 M urea. Fractions were pooled as indicated by the bars. The third pool, step-eluted with EGTA, contained translation calmodulin and was analyzed as described in Figs. 2 and 4.

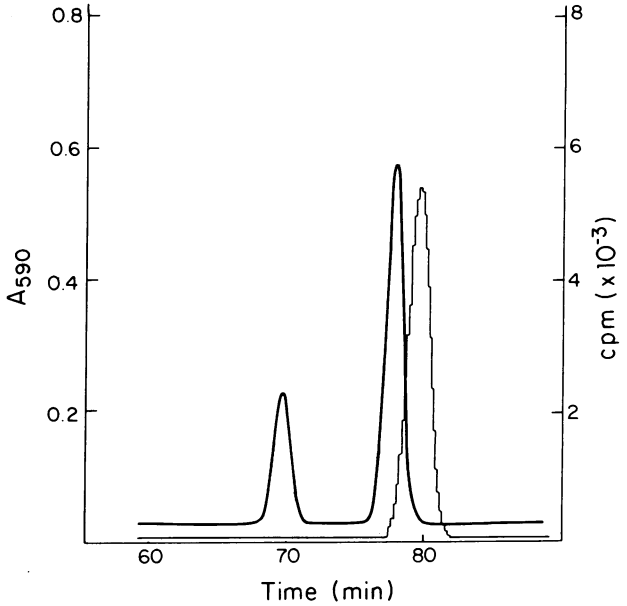

FIG. 4. Determination of trimethyllysine in cell-free translated calmodulin. The analysis was performed as described in the text. The abscissa shows retention time. The thick-lined chromatogram and left-hand ordinate show ninhydrin reactivity. The first peak is trimethyllysine standard and the second peak is lysine standard. The thin-lined histogram and right-hand ordinate represent radioactivity in the sample. The 2-min displacement of the histogram is the delay time between photometer detection and effluent collection.

dependent mobility shift of the same order of magnitude as spinach leaf calmodulin (Fig. 2 *C* and *D*). When the EGTA-eluted material was passed over the phenothiazine-Sepharose column in the presence of EGTA, it did not bind to the column.

To determine if the polypeptide isolated from the translation mixture contained N^{ϵ}-trimethyllysine, an aliquot was acid-hydrolyzed, mixed with lysine and N^{ϵ}-trimethyllysine standard, and subjected to amino acid analysis. The effluent from the photometer of the amino acid analyzer was collected and radioactivity as a function of time after start of analysis was determined. The delay time, approximately 2 min, between the appearance of a photometer peak and its effluent collection was determined by the coinjection of [^{3}H]lysine and lysine standard.

The amino acid analysis of the polypeptide isolated from the translation mixture is shown in Fig. 4. The radioactivity detected in the acid hydrolysate exactly coincided with the lysine standard when adjusted for delay time. No cpm above baseline were detected in the trimethyllysine area of the elution profile. The peak fraction contained 5400 cpm, while the "baseline" fractions contained approximately 40 cpm. Thus, if N^{ϵ}-trimethyllysine had been present at a ratio of 1 trimethyllysyl residue per 20 lysyl residues it would have been detected under these conditions. The ratio of trimethyllysyl to lysyl residues in bovine brain calmodulin is 1 to 7 (3, 9). The ratio in other calmodulins, including spinach leaf calmodulin,* is similar to that found in the bovine brain protein (2, 3, 6–12, 22). There was no evidence of partial methylation—i.e., the presence of N^{ϵ}-dimethyllysine or N^{ϵ}-monomethyllysine—when the hydrolysate was analyzed with the isothermal program. The hydrolysate was also analyzed with the temperature change program described under *Materials and Methods*. Again, the only radioactivity peak detected exactly comigrated with lysine. Together, these two elution systems indicate that the calmodulin translated and isolated as described lacks methyllysine derivatives. When translations were done in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine, radioactivity that comigrated with N^{ϵ}-monomethyllysine was detected.

DISCUSSION

Calmodulin has been produced by translation *in vitro*, isolated by using affinity adsorption procedures, and compared to calmodulins isolated from tissue in terms of various physicochemical and functional properties. There are several salient features of these studies that should be noted. First, the calcium-dependent mobility shift during gel electrophoresis and the calcium-dependent binding to phenothiazines indicate that the translation product had calcium-binding activity and calcium-dependent drug-binding activity similar to calmodulin. It has been reported (23–27) that when phenothiazines are bound to calmodulin in the presence of calcium the ability of calmodulin to activate various enzymes is blocked. Studies using calcium-dependent complex formation between the translated product and calmodulin-regulated proteins are not reported here. The demonstration that trimethylation, which is a co-translational or posttranslational event, is not required for interaction with phenothiazine-Sepharose conjugates shows the potential utility of these conjugates in the study of calmodulin biosynthesis and in studies of calmodulin mRNA and cDNA using *in vitro* translation monitoring.

Second, it should be noted that identical treatment of calmodulin produced by translation and calmodulins isolated from tissues is important in comparative analyses using polyacrylamide gel electrophoresis. It has been reported by Grab *et al.* (28) and by Burgess *et al.*† that the calcium concentration will affect the mobility of calmodulin during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. We report here similar results as well as the utility of this phenomenon in contrasting calmodulins from widely divergent species. The shift in electrophoretic mobility of spinach leaf

calmodulin in the presence of EGTA is not as large as that observed with vertebrate calmodulins. Therefore, a major difference between translation and tissue proteins might be assumed if they differed in their amounts of bound calcium or if translation calmodulin was compared to tissue calmodulin from a phylogenetically distant species. Affinity-based precipitations of translation mixtures by using antiserum to vertebrate calmodulin also demonstrated a major polypeptide that comigrated with calmodulin isolated from the same tissue and species (unpublished observations). More detailed studies, such as NH_2-terminal sequence analysis of the translation product and comparative peptide mapping of the translation and mature spinach protein, are required in order to unequivocally demonstrate that the cell-free translation product is calmodulin.

Third, the translation–purification–characterization system described in this report furnishes the necessary methods for detailed studies of the trimethylation reaction. The ability of various unmethylated calmodulins to be partially or fully methylated by preparations of methylase III, an enzyme capable of generating trimethyllysine from lysine (29), can be directly tested with a relatively homogeneous substrate. The comparative efficiency of methylation during translation or after completion of translation can now be directly studied *in vitro*. The differential effects of methylation on trypsin-catalyzed cleavage at lysyl residue 115 can also be directly tested. Finally, by the use of isotopically labeled amino acids in the translation mixture and the pretreatment of the translation mixture with oxaloacetate and citrate synthase (30), it might be possible to prevent NH_2-terminal acetylation of calmodulins (3) and to directly elucidate the NH_2-terminal sequence of various calmodulins.

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