

## Chemical Synthesis and Expression of a Calmodulin Gene Designed for Site-Specific Mutagenesis<sup>†</sup>

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**ABSTRACT:** A gene coding for a calmodulin was synthesized and expressed in *Escherichia coli*. The gene was produced by the enzymatic ligation of 61 chemically synthesized DNA fragments. The gene possesses 27 unique, regularly spaced, restriction endonuclease cleavage sites to facilitate gene mutagenesis by the replacement of specific gene segments with synthetic double-stranded DNA. An expression vector containing the calmodulin gene was used to transform *E. coli*. Purification and characterization of calmodulin (VU-1 calmodulin) expressed by these transformants showed that it lacks two posttranslational modifications: an amino-terminal blocking group and  $N^{\epsilon},N^{\epsilon},N^{\epsilon}$ -trimethyllysine at position 115. The cyclic nucleotide phosphodiesterase activator properties of VU-1, higher plant, and vertebrate calmodulins were not statistically different. However, VU-1 calmodulin was found to activate nicotinamide adenine dinucleotide (NAD) kinase to a maximal level that was at least 3-fold higher than that found with higher plant and vertebrate calmodulins. This higher level of activation is also characteristic of calmodulins from *Dictyostelium discoideum* and *Chlamydomonas reinhardtii* [Roberts, D. M., Burgess, W. H., & Watterson, D. M. (1984) *Plant Physiol.* 75, 796-798; Marshak, D. R., Clarke, M., Roberts, D. M., & Watterson, D. M. (1984) *Biochemistry* 23, 2891-2899]. The only common feature among *Dictyostelium*, *Chlamydomonas*, and VU-1 calmodulins not found in higher plant and vertebrate calmodulins is an unmethylated lysine at position 115. The results indicate that the lack of methylation of lysine-115 may contribute to the maximal level of NAD kinase activation. These studies demonstrate the feasibility of using a synthetic calmodulin gene product in elucidating the molecular basis of calmodulin action and provide a basis for site-specific mutagenesis studies.

Calmodulin is a highly conserved calcium binding protein that is ubiquitous among eukaryotes. Calmodulin has multiple biochemical activities, including calcium-dependent modulation of a number of enzyme activities and the specific interaction with other nonenzymatic proteins, peptides, and drugs [see Klee & Vanaman (1982), Burgess et al. (1983), Watterson et al. (1984), and Van Eldik & Watterson (1985) for reviews]. The question of how calmodulin structure is related to activity has been addressed by a number of approaches, including chemical modification of specific amino acid residues, analysis of proteolytic and synthetic peptide fragments, cross-linking with bifunctional reagents, immunochemical methods, and analysis of the activity of phylogenetically distinct calmodulins and related calcium binding proteins with well-defined amino acid sequence differences [for recent reviews, see Klee & Vanaman (1982), Watterson et al. (1984), and Van Eldik & Watterson (1985)]. In many cases the study of calmodulin structure and function has been limited by the inability to modify specific amino acid residues within the molecule or by the lack of availability of calmodulins from natural sources with the desired amino acid sequence substitutions. With recent developments in DNA synthesis (Narang, 1983; Wetzel & Goeddel, 1983), it is possible to generate a functional gene from oligodeoxynucleotides that (1) codes for the desired protein and (2) contains unique restriction endonuclease recognition sites for the introduction of specific modifications

in the coding sequence. The availability of such a gene coding for calmodulin would provide a system for the generation of calmodulins with specific modifications for further studies on the relationship of structure to activity. In this report we describe the design, chemical synthesis, cloning, and expression of a calmodulin gene and the characterization of the protein product (VU-1 calmodulin).<sup>1</sup>

### MATERIALS AND METHODS

**Deoxyoligonucleotides.** The chemical synthesis of oligonucleotides was performed by a phosphotriester method as described by Crea & Horn (1980) or by an automated phosphite triester method as described below. Oligonucleotides calmo-1-31 (Figure 1) were synthesized from dinucleotide reagents on a cellulose support by the phosphotriester method (Crea & Horn, 1980). Oligonucleotides calmo-32-61 (Figure 1) were synthesized from diisopropylphosphoramidite nucleotides (Matteucci & Caruthers, 1981) by using the automated stepwise addition protocol of Alvarado-Urbina et al. (1981). 5'-(Dimethoxytrityl)-2'-deoxynucleosides (1 mmol) were converted into the corresponding diisopropylphosphoramidite derivatives in reaction mixtures containing 15 mL of anhydrous acetonitrile, 0.6 mL of dry 2,6-lutidine, and 0.2 mL

<sup>1</sup> Abbreviations: pVUC-1 (Vanderbilt University calmodulin 1), plasmid vector composed of pKK223-3 (P-L Biochemicals; Amann et al., 1983) and the synthetic calmodulin gene; VU-1 calmodulin, expressed product of pVUC-1; IPTG, isopropyl  $\beta$ -D-thiogalactoside; Pth, phenylthiohydantoin; DTT, dithiothreitol; BSA, bovine serum albumin; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid; trimethyllysine,  $N^{\epsilon},N^{\epsilon},N^{\epsilon}$ -trimethyllysine; calmo, calmodulin; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide; PEG, poly(ethylene glycol); Tris, tris(hydroxymethyl)aminomethane; Tris-HCl, Tris hydrochloride.

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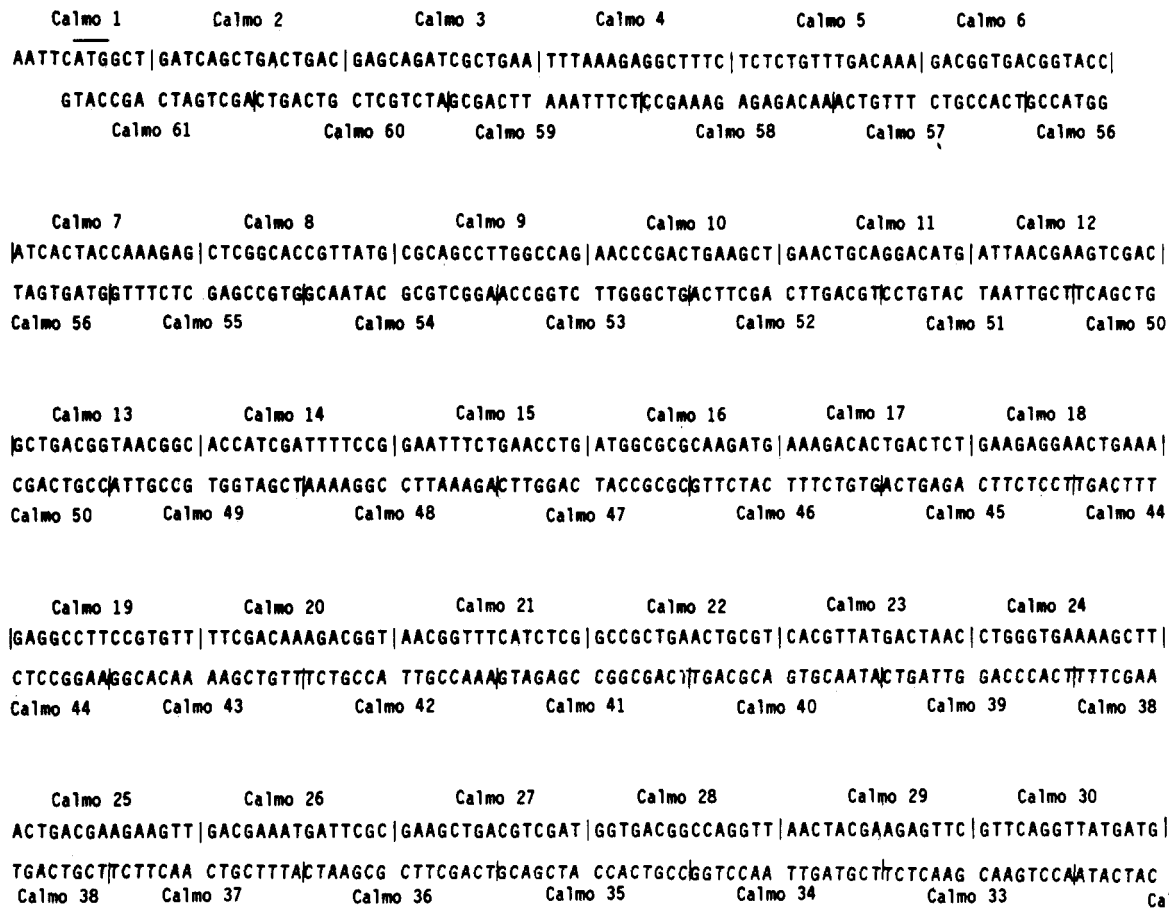
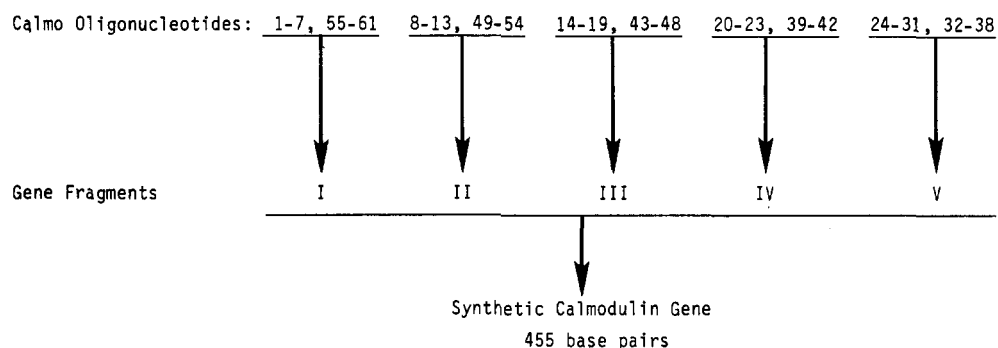


FIGURE 1: Nucleotide sequence of the synthetic calmodulin gene. The sequence of the calmodulin gene and the corresponding positions of the 61 synthetic oligonucleotide fragments are shown. Bars over the sequences in oligonucleotides calmo-1 and calmo-31 correspond to the translation initiation and termination codons, respectively. The single-strand sequences at the 5'-termini of oligonucleotides calmo-1 and calmo-32 are complementary to cohesive ends generated in the vector DNA by digestion with *EcoRI* and *BamHI*, respectively.



**FIGURE 2: Synthetic scheme for the calmodulin gene.** The complementary oligonucleotides indicated were combined and were enzymatically phosphorylated and ligated as described under Materials and Methods to produce gene fragments I–V. The calmodulin gene was generated from the purified gene fragments by enzymatic ligation.

of chloro(*N,N*-diisopropylamino)methoxyphosphine. After 15 min of shaking, 30 mL of 7 mg/mL 1*H*-tetrazole in acetonitrile was added to the reaction mixture. The resulting activated phosphoramidite derivatives were used for oligonucleotide synthesis on a derivatized silica support (Alvarado-Urbina et al., 1981). A typical addition cycle consisted of (a) addition of phosphoramidite derivative (1 min), (b) stop flow (1 min), (c) addition of 1% iodine in tetrahydrofuran-pyridine-water (3:1:1 v/v) (30 s), (d) pyridine wash (1.5 min), (e) methylene chloride wash (1 min), (f) wash with 3% trichloroacetic acid in methylene chloride (v/v) (1.5 min), (g) methylene chloride wash (1.5 min), and (h) acetonitrile wash (2 min). All steps were carried out by using an automated stop-flow system designed at Creative Biomolecules, Inc. (Hopkinton, MA). The flow rate was maintained at 5

mL/min for the entire cycle.

At the completion of synthesis, oligonucleotides were treated with dioxane-triethylamine-thiophenol (2:1:1 v/v) at room temperature for 45 min and then with concentrated ammonia at 55 °C overnight. The oligonucleotides were purified from the resulting mixture by thin-layer chromatography on Kieselgel 60 plates (Alvarado-Urbina et al., 1981). The purity and size of the final products were confirmed by electrophoretic analysis on polyacrylamide gels as described previously (Crea et al., 1978).

**Calmodulin Gene Ligation.** The calmodulin gene was constructed from 61 oligonucleotides as shown in Figures 1 and 2. Five large gene fragments, I-V, were synthesized from oligonucleotides by 5'-phosphorylation followed by enzymatic ligation. Phosphorylation was carried out at 37 °C for 1 h

in a reaction mixture containing oligonucleotides (1.2  $\mu\text{g}$  of each), 1 mM ATP, T4 phosphorylase kinase (1.3 units/ $\mu\text{g}$  of DNA), 10 mM  $\text{MgCl}_2$ , 5 mM dithiothreitol, and 70 mM Tris-HCl, pH 7.6. Ligation of the phosphorylated oligonucleotides was carried out at 15  $^\circ\text{C}$  for 2 h in a reaction mixture containing 0.75 mM ATP, T4 DNA ligase (1.5 units/ $\mu\text{g}$  of DNA), 10 mM  $\text{MgCl}_2$ , 20 mM dithiothreitol, 50  $\mu\text{g}/\text{mL}$  BSA, and 50 mM Tris-HCl, pH 7.8. The DNA fragments were resolved by electrophoresis on 8% (w/v) polyacrylamide gels in the Tris-borate-EDTA buffer system described by Maniatis et al. (1975). Bands migrating at the expected molecular weight were sliced from the gel and were electroeluted (Maniatis et al., 1982). The eluted DNA was taken to dryness under vacuum and was resuspended in 200  $\mu\text{L}$  of 0.2 M sodium acetate, pH 5. The sample was extracted twice with an equal volume of phenol, chloroform, and isoamyl alcohol (50:50:1) and once with chloroform, and the DNA was precipitated with 2.5 volumes of absolute ethanol. The purified gene fragments were stored at 4  $^\circ\text{C}$  in 1 mM Tris-HCl and 0.1 mM EDTA, pH 7.5.

Stoichiometric amounts of gene fragments I-V (5.4  $\mu\text{g}$  total) were combined and ligated by using the conditions described above. After gel electrophoresis, the DNA band that migrated at 455 base pairs was sliced from the gel, electroeluted, and purified as described above.

**Amplification Cloning of the Calmodulin Gene.** The synthetic calmodulin gene was inserted into the *EcoRI* and *BamHI* sites of pUC8 (Viera & Messing, 1982). pUC8 (6  $\mu\text{g}$ ) was digested at 37  $^\circ\text{C}$  in a 40- $\mu\text{L}$  reaction mixture containing *BamHI* (32 units), 6 mM  $\text{MgCl}_2$ , 150 mM NaCl, 100  $\mu\text{g}/\text{mL}$  BSA, and 6 mM Tris-HCl, pH 7.9. After 1 h, 6  $\mu\text{L}$  of 1 M Tris-HCl, pH 7.5, and 40 units of *EcoRI* were added to the digestion mixture. The volume was adjusted to 60  $\mu\text{L}$  with sterile water, and the digestion was allowed to proceed at 37  $^\circ\text{C}$  for an additional 1 h. The DNA fragments were resolved by electrophoresis on a 6% (w/v) polyacrylamide gel. The large fragment was sliced from the gel and was electroeluted. The synthetic calmodulin gene (30 ng) and the large *EcoRI/BamHI* fragment of pUC8 (100 ng) were combined and treated with T4 DNA ligase. The ligation mixture was used to transform competent *Escherichia coli* K12 UT481 cells. Competent cells were prepared by using the low-pH method described by Enea et al. (1975). Transformants were selected by plating on NZCYM agar (Maniatis et al., 1982) containing 25  $\mu\text{g}/\text{mL}$  ampicillin. Plasmids were isolated from small cultures of transformed bacteria by using a modification of the method of Birnboim & Doly (1979) as described by Maniatis et al. (1982). Purified plasmids were screened for the presence of the 455 base pair calmodulin gene insert by *EcoRI* and *BamHI* digestion, followed by polyacrylamide gel electrophoresis. Large-scale preparations of plasmids containing the calmodulin gene insert were done by using the alkaline lysis method of Birnboim & Doly (1979).

**DNA Sequence Analyses.** The DNA sequence of the cloned calmodulin gene was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977). pUC8 plasmid containing the calmodulin gene was cleaved with *EcoRI* and *BamHI*, and the gene insert was purified by gel electrophoresis. The calmodulin gene was inserted into the *EcoRI* and *BamHI* sites of M13 mp10w and mp11w (New England Biolabs; Messing, 1983). Single-stranded M13 templates were prepared by using the method of Schreier & Cortese (1979).

**Calmodulin Gene Expression.** An expression vector containing the calmodulin gene (pVUC-1) was constructed as

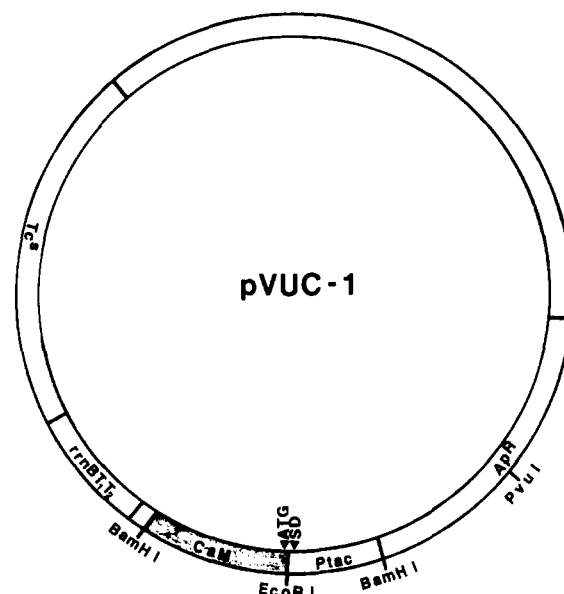


FIGURE 3: Structure of pVUC-1 calmodulin expression vector. Construction of pVUC-1 from pKK223-3 and the synthetic calmodulin gene is discussed under Materials and Methods. Sequences derived from the pKK223-3 vector are unshaded, and sequences derived from the calmodulin gene (CaM) are shaded. *Pac* refers to the hybrid trp-lac promoter (Amann et al., 1983) and *rrnBT12* refers to a fragment from the ribosomal RNA operon *rrnB* that contains two transcriptional terminator signals *T1* and *T2* (Brosius et al., 1981). The position of the Shine-Dalgarno sequence (SD) relative to the initiation codon (ATG) of the calmodulin gene is indicated by  $\blacktriangledown$ . *Ap<sup>R</sup>* and *Tc<sup>R</sup>* refer to the ampicillin resistance marker and a fragment of the tetracycline resistance marker from pBR322 (Bolivar et al., 1977). All regions of the plasmid are drawn to scale assuming that pVUC-1 is 5030 base pairs.

shown in Figure 3. The calmodulin gene was inserted into the *EcoRI* and *BamHI* sites of the expression plasmid pKK223-3 (P-L Biochemicals; Aman et al., 1983). pKK223-3 (0.75  $\mu\text{g}$ ) was digested at 37  $^\circ\text{C}$  for 1 h in a reaction mixture containing 20 units of *EcoRI*, 4 units of *PvuI*, 10 mM  $\text{MgCl}_2$ , 50 mM NaCl, 1 mM dithiothreitol, 100  $\mu\text{g}/\text{mL}$  BSA, and 50 mM Tris-HCl, pH 8. The DNA fragments were resolved by agarose gel electrophoresis, and the 3500 base pair *EcoRI/PvuI* fragment was eluted and purified by using the method of Hong (1982). A separate digest was performed with the pKK223-3 vector by using the same conditions described above with the exception that 16 units of *BamHI* were used instead of *EcoRI*. After gel electrophoresis, the 1000 base pair *PvuI/BamHI* fragment was eluted and purified. Stoichiometric amounts of the *EcoRI/PvuI* and *BamHI/PvuI* fragments of pKK223-3 (350 ng total DNA) were combined with the calmodulin gene (90 ng) and were treated with 6 units of T4 DNA ligase for 2 h at 15  $^\circ\text{C}$  in a reaction mixture containing 1 mM ATP, 10 mM  $\text{MgCl}_2$ , 20 mM DTT, 50  $\mu\text{g}/\text{mL}$  BSA, and 20 mM Tris-HCl, pH 7.8. The ligation mixture was used to transform competent *E. coli* K12 UT481 cells. Transformants were selected by plating on NZCYM agar containing 25  $\mu\text{g}/\text{mL}$  ampicillin.

Transformants were screened for the production of calmodulin. Aliquots (1 mL) of NZCYM medium containing 25  $\mu\text{g}/\text{mL}$  ampicillin were inoculated with single colonies of transformed bacteria and were incubated overnight at 37  $^\circ\text{C}$  with shaking. The overnight cultures were transferred to 3 mL of NZCYM medium containing 1.33 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG) and were grown at 37  $^\circ\text{C}$  for 2 h. The cells in 1 mL of the cultures were collected by centrifugation. The cell pellet was resuspended in 200  $\mu\text{L}$  of 4 mg/mL lysozyme (Sigma), 50 mM Tris-HCl, pH 7.5, 2 mM EDTA,

and 1 mM DTT and was incubated at 0 °C for 1 h. The samples were heated at 80 °C for 6 min and were centrifuged. The supernatant was tested for calmodulin by NAD kinase (Roberts et al., 1984) or cyclic nucleotide phosphodiesterase (Watterson et al., 1980a) activator assays.

**Purification of Expressed Calmodulin (VU-1 Calmodulin).** Bacterial clones that were found to express calmodulin were cultured overnight at 37 °C in 50 mL of NZCYM medium containing 25 µg/mL ampicillin. The NZCYM medium (1100 mL) was inoculated with 25 mL of the overnight culture and was incubated at 37 °C with shaking. When the  $A_{550}$  of the culture reached 0.4, IPTG was added to a final concentration of 1 mM, and growth was continued for an additional 2 h. The cells were collected by centrifugation at 8000g for 10 min, and the cell pellet was washed with 50 mM Tris-HCl, pH 7.5. The washed cells were lysed by using a modification of the method of Stein et al. (1984). The cell pellet was suspended in 50 mL of 200 µg/mL lysozyme, 2 mM EDTA, 1 mM DTT, and 50 mM Tris-HCl, pH 7.5, and was incubated at 0 °C for 30 min. DNase and MgCl<sub>2</sub> were added to the digestion mixture to final concentrations of 180 units/mL and 3 mM, respectively, and the mixture was incubated at 0 °C for an additional 20 min. The mixture was centrifuged at 27000g for 30 min. The supernatant was decanted, and a saturated solution of ammonium sulfate was slowly added to produce a 55% saturated solution. The solution was stirred at 4 °C for 30 min and was centrifuged at 27000g for 20 min. The supernatant was adjusted to pH 4 by the addition of 50% (v/v) H<sub>2</sub>SO<sub>4</sub>. The solution was stirred for 30 min at 4 °C and was centrifuged at 27000g for 20 min. The pellet was suspended in 5 mL of 50 mM Tris-HCl, pH 7.5, and 1 mM DTT and was dialyzed against 20 L of water for 1 h and then overnight against 2 L of 10 mM Tris-HCl, 0.1 M NaCl, 0.5 mM EGTA, and 1 mM DTT, pH 7.5. The particulate matter was removed from the dialyzed sample by centrifugation at 27000g for 15 min. VU-1 calmodulin was purified from the resuspended pH 4 pellet by chromatography on phenyl-Sepharose as described by Gopalakrishna & Anderson (1982). CaCl<sub>2</sub> was added to a final concentration of 5 mM, and the sample was applied to a 1-mL column of phenyl-Sepharose (Pharmacia). The column was washed with 50 mM Tris-HCl, 0.5 mM DTT, and 0.1 mM CaCl<sub>2</sub>, pH 7.5, until the absorbance at 280 nm was less than 0.01. The column was then washed with 50 mM Tris-HCl, 0.5 M NaCl, 0.1 mM CaCl<sub>2</sub>, and 0.5 mM DTT, pH 7.5, until there was no detectable absorbance at 280 nm. Calmodulin was eluted from the column with 50 mM Tris, 0.5 mM DTT, and 1 mM EGTA, pH 7.5. The eluant was dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub> and then extensively against deionized water. Purified VU-1 calmodulin was stored frozen at -80 °C.

**Protein Chemistry Methods.** Amino acid analyses were performed as described previously (Marshak et al., 1984). Analysis of trimethyllysine was done as described by Van Eldik et al. (1980).

Amino acid sequence determinations were done by automated Edman degradation using an Applied Biosystems Model 470A sequencer and by the procedure of Hewick et al. (1981) as described by Marshak et al. (1984). Identification of Pth-amino acids was done by high-performance liquid chromatography on a Zorbax (Du Pont) (octadecylsilanyl)silica column (4.1 mm × 250 mm) by using the method of Zimmerman et al. (1977) as described by Marshak et al. (1984). Repetitive yields of all reported sequencer runs were above 92%.

Digestion of VU-1 calmodulin with the arginine-specific protease from mouse submaxillary gland (Arg proteinase C, Boehringer-Mannheim) was done by using a previously described protocol (Marshak et al., 1984; Lukas et al., 1984). Ten nanomoles of VU-1 calmodulin was digested at 37 °C for 22 h in a mixture (50 µL) containing 85 mM NH<sub>4</sub>HCO<sub>3</sub>, 1 mM EGTA, and 100 µg/mL protease. Peptides were resolved by reverse-phase high-performance liquid chromatography on an (octyldecylsilanyl)silica column (Whatman ODS-3, 10 mm × 250 mm) by using the general procedures of Lukas et al. (1984) and Marshak et al. (1984).

**Preparation of NAD Kinase.** NAD kinase was extracted and partially purified from pea seedlings by using modifications of previously described protocols (Muto & Miyachi, 1977; Jarrett et al., 1982; Harmon et al., 1984). Two week old seedlings (390 g) were homogenized in 1.2 L of 25 mM triethanolamine hydrochloride, 1 mM phenylmethanesulfonyl fluoride, and 0.5% (w/v) poly(vinylpyrrolidone), pH 7.5. The homogenate was squeezed through three layers of cheesecloth and was centrifuged at 11000g for 30 min at 4 °C. NAD kinase in the supernatant was purified through the protamine sulfate and poly(ethylene glycol) (PEG) precipitation steps as described by Muto & Miyachi (1977). The PEG-precipitated enzyme was resuspended in 50 mM Tris-HCl, 100 mM KCl, 3 mM MgCl<sub>2</sub>, and 0.2 mM EGTA, pH 7.5, and was centrifuged for 15 min at 27000g to remove insoluble material. The supernatant was passed through a DEAE-Sephadex A-25 column (1.5 cm × 5 cm). The effluent was collected, and CaCl<sub>2</sub> was added to a final concentration of 1 mM. The enzyme was adsorbed to a 3-mL calmodulin-Sepharose column prepared essentially as previously described (Watterson & Vanaman, 1976), and the column was washed with 25 mM Tris-HCl, 0.2 M KCl, 3 mM MgCl<sub>2</sub>, and 0.5 mM CaCl<sub>2</sub>, pH 8, until the absorbance at 280 nm was less than 0.02. The column was then washed with 25 mM Tris-HCl, 0.5 M KCl, 3 mM MgCl<sub>2</sub>, and 0.5 mM CaCl<sub>2</sub>, pH 8, and was eluted with 25 mM Tris-HCl, 0.2 M KCl, 3 mM MgCl<sub>2</sub>, and 1 mM EGTA, pH 8. All purification steps were performed within a 10-h period. We obtained an enzyme preparation with a specific activity of 700–800 nmol of NADP produced min<sup>-1</sup> (mg of protein)<sup>-1</sup> when tested with a saturating amount (40 pmol) of spinach calmodulin. We found that the time span of the NAD kinase preparation protocol is important for obtaining an enzyme with the activator properties previously described (Roberts et al., 1984; Marshak et al., 1984). In particular, if the eluant from the DEAE-Sephadex step is left at 4 °C for as short as 2–3 h, the difference between the maximal activation obtained with spinach and *Chlamydomonas* calmodulins is no longer observed. The purified enzyme was stored at -80 °C in 5% (v/v) glycerol as described by Harmon et al. (1984).

**General Methods.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of protein samples was done by using the method of Laemmli (1970). Calmodulins from chicken gizzard, *Chlamydomonas*, and spinach were extracted and purified as described previously (Van Eldik & Watterson, 1979; Burgess et al., 1980; Watterson et al., 1980c; Schleicher et al., 1984; Lukas et al., 1984). Protein was determined by the method of Lowry et al. (1951). The concentrations of purified calmodulins were determined by amino acid composition analysis.

## RESULTS

The 455 base pair calmodulin gene was constructed from 61 oligonucleotides as shown in Figure 1. The gene was designed with the following considerations: (1) the gene codes



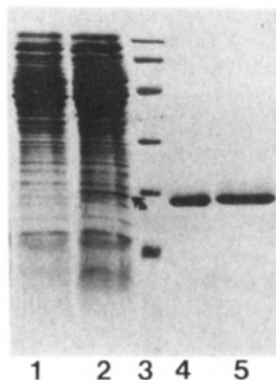


FIGURE 6: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of VU-1 calmodulin. Electrophoresis was performed on 15% (w/v) polyacrylamide gels in the presence of 1 mM EDTA. Lane 1, pH 4 pellet fraction from *E. coli* transformed with pKK223-3; lane 2, pH 4 pellet fraction from *E. coli* transformed with pVUC-1; lane 3, molecular weight standards phosphorylase B ( $M_r$  94 000), BSA ( $M_r$  67 000), ovalbumin ( $M_r$  43 000), carbonic anhydrase ( $M_r$  30 000), soybean trypsin inhibitor ( $M_r$  20 100), and  $\alpha$ -lactalbumin ( $M_r$  14 400); lane 4, chicken gizzard calmodulin; lane 5, VU-1 calmodulin after phenyl-Sepharose chromatography. The arrow in lane 2 indicates the protein band in the pH 4 pellet obtained from bacteria transformed with pVUC-1 that is similar in electrophoretic mobility to chicken gizzard calmodulin.

at least 0.4% of the total soluble protein on the basis of cyclic nucleotide phosphodiesterase activator analyses. In the control experiment, bacteria transformed with pKK223-3 were used. As indicated in Table I, calmodulin activity was not detected in the bacterial extract or in the pH 4 pellet obtained from the control bacteria. Similar results were obtained when NAD kinase activator analyses were performed (data not shown). The calmodulin activity present in extracts of bacteria containing pVUC-1 could be purified (Table I) by established calmodulin purification protocols (Watterson et al., 1976; Gopalakrishna & Anderson, 1982).

Sodium dodecyl sulfate gel electrophoresis of the pH 4 pellet fraction obtained from a bacterial clone that expressed calmodulin activity showed the presence of a protein band with an electrophoretic mobility similar to that of vertebrate calmodulin (Figure 6). This band was not apparent in the electrophoretic profile of the pH 4 pellet from control bacterial clones (Figure 6). This protein, VU-1 calmodulin, can be purified by calcium-dependent chromatography of the resolubilized pH 4 pellet on phenyl-Sepharose (Figure 6).

VU-1 calmodulin was characterized further by amino acid composition and limited amino acid sequence analyses. Amino acid composition analysis of acid hydrolysates of VU-1 calmodulin (Table II) demonstrates that it has the amino acid composition expected on the basis of the amino acid sequence coded by the synthetic gene. In particular, the nine lysine residues predicted from the amino acid sequence coded by the gene were found, and trimethyllysine was not detected. Edman degradation of VU-1 calmodulin resulted in the quantitative release of alanine in the first cycle and yielded 21 additional cycles of amino acid derivatives corresponding to residues 2–22 of VU-1 calmodulin (Figure 4). In addition to verifying the integrity of the amino-terminal region, the sequence data show that VU-1 calmodulin lacks the amino-terminal formyl-methionine residue coded by the ATG initiation codon and that the amino-terminal alanine residue does not have a blocking group.

Recent work (Schleicher et al., 1984; Marshak et al., 1984; Lukas et al., 1984) has shown that calmodulin can be digested with an arginine-specific protease to yield a carboxyl-terminal peptide (residues 107 to the carboxyl terminus) that can be

Table II: Amino Acid Composition of VU-1 Calmodulin

| amino acid                       | VU-1 calmodulin (mol/17 000 g) | amino acid    | VU-1 calmodulin (mol/17 000 g) |
|----------------------------------|--------------------------------|---------------|--------------------------------|
| Asp                              | 27.0 (24) <sup>a</sup>         | Ile           | 6.0 (6)                        |
| Thr                              | 9.7 (10)                       | Leu           | 11.5 (11)                      |
| Ser                              | 4.2 (4)                        | Tyr           | 0.9 (1)                        |
| Glu                              | 28.0 (28)                      | Phe           | 8.7 (9)                        |
| Pro                              | 2.0 (2)                        | His           | 1.1 (1)                        |
| Gly                              | 11.6 (11)                      | trimethyl-Lys | 0                              |
| Ala                              | 11.5 (11)                      | Lys           | 8.8 (9)                        |
| <sup>1</sup> / <sub>2</sub> -Cys | nd (0) <sup>b</sup>            | Trp           | nd (0)                         |
| Val                              | 9.2 (9)                        | Arg           | 4.7 (5)                        |
| Met                              | 7.7 (8)                        |               |                                |

<sup>a</sup>Residues per molecule based on the predicted amino acid sequence of VU-1 calmodulin (Figure 4) are given in parentheses. <sup>b</sup>nd, not determined.

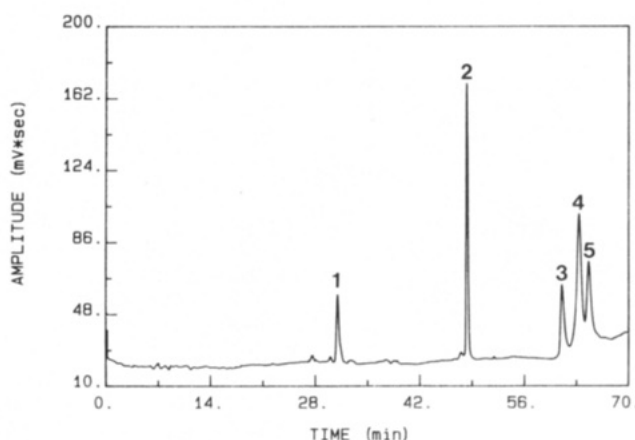


FIGURE 7: Separation of peptides generated by digestion of VU-1 calmodulin with the arginine-specific protease. Digestion conditions are described under Materials and Methods. An aliquot of the digest (1.6 nmol of VU-1 calmodulin) was applied to a column (10 mm  $\times$  250 mm) of (octadecylsilyl)silica equilibrated in 95% (v/v) 0.01 N HCl (solvent A) and 5% (v/v) acetonitrile (solvent B). The peptides were eluted with the following programmed changes in solvent B: 5 min, 5% B; 10 min, 10% B; 20 min, 25% B; 30 min, 25% B; 40 min, 35% B; 50 min, 35% B; 60 min, 45% B; 70 min, 45% B. The ordinate is the detector signal output at 215 nm.

isolated in high yield by high-performance liquid chromatography. Edman degradation of this peptide has been done with other calmodulins to establish the presence of lysine at position 115 when the lack of trimethyllysine is indicated from the amino acid composition (Marshak et al., 1984; Schleicher et al., 1984). Digestion of VU-1 calmodulin with the arginine-specific protease results in the production of a peptide (peak 2, Figure 7) with chromatographic properties similar to those of the carboxyl-terminal peptides from other calmodulins. Edman degradation of this peptide generated 15 cycles of Pth-amino acids identical with those expected for residues 107–121 of VU-1 calmodulin (Figure 4). Cycle nine (position 115) was lysine. This result supports amino acid composition analyses and shows that position 115 of VU-1 calmodulin is a lysine.

The cyclic nucleotide phosphodiesterase and the NAD kinase activator properties of VU-1 calmodulin also were investigated. Assays were done in conditions (1 mM  $\text{CaCl}_2$ ) that should result in the saturation of all calcium binding sites of calmodulin. VU-1 calmodulin did not activate either enzyme when 1 mM EGTA was substituted for  $\text{CaCl}_2$  in the assay. VU-1 calmodulin activated phosphodiesterase to the same extent as vertebrate and higher plant calmodulins. In multiple assays the concentrations of each calmodulin required for half-maximal activation of phosphodiesterase ranged between

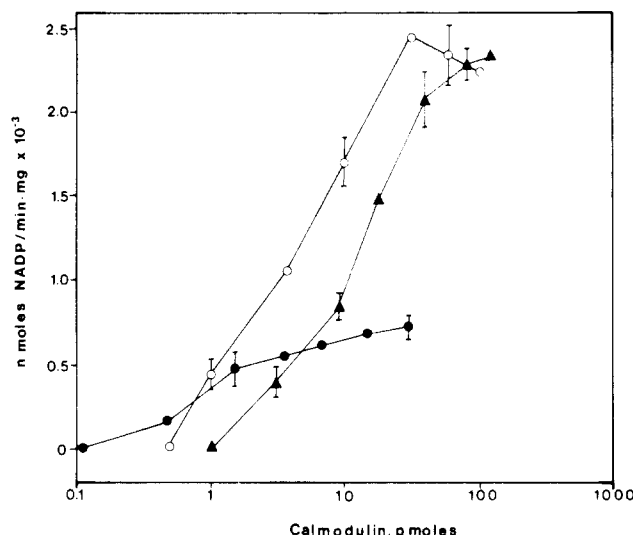


FIGURE 8: NAD kinase activator activities of VU-1, *Chlamydomonas*, and spinach calmodulins. Assays were performed essentially as previously described (Roberts et al., 1984). Each assay mixture (0.5 mL) consisted of 2 mM NAD, 3 mM ATP, 1 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 50 mM Tris-HCl, pH 8, NAD kinase (10  $\mu$ L of a 11.5 nmol min<sup>-1</sup> mL<sup>-1</sup> preparation), and various amounts of spinach (●), VU-1 (○), and *Chlamydomonas* (▲) calmodulins. Incubations were carried out at 37 °C for 20 min. The reaction was terminated by placing samples in a boiling water bath for 3 min. Each point is the average of two determinations. The error bars show the range of values obtained. The absence of error bars indicates that the range is smaller than the figure symbol.

8 and 14 nM for spinach and gizzard calmodulins and between 9 and 18 nM for VU-1 calmodulin (data not shown).

In contrast to the phosphodiesterase activator results, VU-1 calmodulin activated NAD kinase to a maximal level 3.2-fold higher than that obtained with spinach calmodulin (Figure 8) and 6.0-fold higher than that obtained with gizzard calmodulin (data not shown). The maximum level of NAD kinase activation obtained with VU-1 calmodulin is similar to that observed with *Chlamydomonas* (Figure 8; Roberts et al., 1984) and *Dictyostelium* (Marshak et al., 1984) calmodulins. In addition to differences in maximal activation, VU-1 calmodulin differed from other calmodulins in the degree of activation obtained with submaximal calmodulin concentrations. The amounts of VU-1, *Chlamydomonas*, and vertebrate calmodulin required to yield the NAD kinase activation [360 nmol of NADP min<sup>-1</sup> (mg of protein)<sup>-1</sup>] obtained with 1 pmol of spinach calmodulin are 0.9, 2.6, and 25 pmol, respectively (Figure 8; Roberts et al., 1984). Thus, although the sequence of VU-1 differs from that of spinach calmodulin at five positions (Figure 4) and lacks an amino-terminal blocking group, it resembles spinach calmodulin in its activation of NAD kinase at nonsaturating concentrations.

## DISCUSSION

We have designed, synthesized, and obtained expression of a calmodulin gene as part of a continuing study of the relationship between calmodulin structure and function. The synthetic gene was designed to code for a calmodulin that is not known to exist in nature but is a conservative hybrid of two known structures, higher plant and vertebrate calmodulins, that are indistinguishable in terms of phosphodiesterase and myosin light chain kinase activator activities but have readily distinguishable NAD kinase activator properties (Roberts et al., 1984). Comparison of the enzyme activator activities of the expressed product of the gene, VU-1 calmodulin, with several eukaryotic calmodulins has provided further insight

into the relationship of calmodulin structure to activity. In addition, the design of the gene allows for further studies of calmodulin structure and function by gene mutagenesis through the specific incorporation of synthetic DNA fragments.

The coding sequence of the gene corresponds to the amino acid sequence of spinach calmodulin except for the presence of an initiator methionine, threonine-26, glycine-96, valine-136, and glutamine-143. Although a lysine codon was used for amino acid sequence position 115, it was not known in advance whether the *E. coli* host strain would methylate this position. Regardless, on the basis of available data for naturally occurring calmodulins (Marshak et al., 1984; Roberts et al., 1984), the methylation state of lysine-115 does not appear to drastically alter phosphodiesterase or myosin light chain kinase activator properties. Similarly, variations in amino acid sequence at positions 26, 96, 136, and 143 and small variations in the length of the amino terminus among naturally occurring calmodulins do not result in major differences in these calcium-dependent activator activities. The phosphodiesterase activation data actually obtained with VU-1 calmodulin confirm these assumptions.

Certain technical advantages also were considered in the design of the coding sequence at positions 26, 96, 136, and 143. The use of threonine-26 instead of cysteine-26 avoids the technical problems of oxidation or alkylation often encountered in proteins with cysteines that are not found in a disulfide linkage. The presence of valine-136 and glutamine-143 instead of isoleucine-136 and lysine-143 allows the use of site-directed antibodies (Van Eldik et al., 1983) for immunological screening. This becomes an important consideration if future mutagenesis studies result in the production of a calmodulin that has lost activator activity. Glycine is found at position 96 in all calmodulins characterized to date except for higher plant calmodulins, which contain a glutamine. Although it is not clear how a glycine to glutamine change would affect calmodulin structure and calcium binding activity, it may decrease the flexibility of the polypeptide chain in the third calcium binding loop, which may result in altered calcium binding. This is clearly a site for future modification studies. In this regard, it should be noted that the activity comparisons reported in this study were done in the presence of saturating concentrations of calcium, i.e., concentrations well above the known dissociation constants of calmodulin for calcium. On the basis of precedents [for a review, see Klee & Vanaman (1982)], the calmodulins compared in this study are not expected to have major differences in calcium binding activity, but detailed comparative calcium binding studies have yet to be done.

In addition to the calmodulin coding sequence, other considerations that offer advantages over available calmodulin cDNA structures (Lagace et al., 1983; Putkey et al. 1983; Chien & Dawid, 1984) were taken into account in the design of the synthetic gene. Unlike the cDNA structures, the synthetic calmodulin gene possesses *Eco*RI and *Bam*HI cohesive ends to facilitate insertion into plasmid vectors and has 25 additional unique and regularly spaced restriction endonuclease cleavage sites within the coding sequence. The restriction endonuclease cleavage sites within the gene will be particularly important for future site-specific mutagenesis studies. These studies will allow the removal of specific gene segments and the introduction of synthetic DNA fragments with the desired coding changes. In addition, the synthetic calmodulin gene does not have the extended untranslated 5'-region characteristic of calmodulin cDNA structures. The absence of an extended

untranslated 5'-region is important since the proper position of the initiation codon of the gene relative to the bacterial ribosome binding site appears to be necessary for efficient mRNA translation (Shine & Dalgarno, 1975).

*E. coli*, an organism that lacks an endogenous calmodulin (Burgess et al., 1980), was selected as a host for the direct expression of the gene. Expression of the calmodulin gene results in the production of a protein product, VU-1 calmodulin, with calcium-dependent enzyme activator activities. Amino acid sequence analysis of purified VU-1 calmodulin shows that it has an unblocked alanine residue at its amino terminus rather than the methionine residue coded by the initiation codon of the gene. The characterization of protein products from the expression of other foreign genes in *E. coli* indicates that the removal of the amino-terminal formyl-methionine group is variable and seems to depend on the nature of the foreign protein (Wetzel & Goeddel, 1983). For example, expression of the human growth hormone gene results in the production of a protein with an amino-terminal methionine (Stebbing et al., 1981), whereas 50% of the expressed interferon  $\alpha$ -A molecules lack this amino-terminal residue (Wetzel et al., 1981; Staehelin et al., 1981). The presence of an unblocked alanine residue at the amino terminus of VU-1 calmodulin distinguishes it from all calmodulins that have been characterized. The function of acetylated amino termini or other amino-terminal blocking groups in protein activity is not well understood (Wold, 1981). The observation that VU-1 possesses NAD kinase and phosphodiesterase activator activities suggests that an amino-terminal blocking group is not necessary for these calmodulin activities.

In addition to the absence of an amino-terminal blocking group, VU-1 calmodulin lacks trimethyllysine at position 115. Previous studies of the structure and function of *Chlamydomonas* and *Dictyostelium* calmodulins showed that these calmodulins, like VU-1 calmodulin, have an unmodified lysine at position 115 and activate NAD kinase to a maximal level that is at least 3-fold higher than that found with higher plant or vertebrate calmodulins. However, in addition to the lack of trimethyllysine at position 115, *Dictyostelium* and *Chlamydomonas* calmodulins have several similarities not found in higher plant and vertebrate calmodulins: (1) amino acid changes at positions 81, 118, and 148 and (2) the presence of additional amino acids at their carboxyl termini (Figure 4). The fact that VU-1 is identical with higher plant and vertebrate calmodulins at positions 81, 118, and 148 and does not have additional amino acids at its carboxyl terminus indicates that these changes alone cannot account for the higher maximal NAD kinase activator properties. A comparison of amino acid sequences (Figure 4) reveals that *Chlamydomonas*, *Dictyostelium*, and VU-1 calmodulins possess one common feature that distinguishes them from vertebrate and higher plant calmodulins: the lack of trimethyllysine-115. Thus, the data suggest that NAD kinase is activated to a higher maximal level as the result of structural changes in the calmodulin molecule conferred by the absence of methylation. Although purified *N*-methyltransferases that can utilize unmethylated calmodulin as an effective substrate are available to test this hypothesis, these enzymes also incorporate additional methyl groups into calmodulins that already have trimethyllysine at position 115 (Sitaramayya et al., 1980). Current studies (D. M. Roberts, D. M. Watterson, P. Rowe, and F. Siegel, unpublished results) on the role of lysine-115 in NAD kinase activation include attempts to define conditions so that lysine-115 is the only position that is trimethylated. If methylation of lysine-115 does affect NAD kinase activator activity,

the structural alteration is probably subtle because phosphodiesterase and myosin light chain kinase activator activities are not similarly affected by this modification (Schleicher et al., 1984; Marshak et al., 1984; Roberts et al., 1984).

These results with the calmodulin gene product VU-1 and naturally occurring calmodulins have provided new information regarding the potential importance of certain structural features of the calmodulin molecule in the activation of NAD kinase. The availability of the synthetic calmodulin gene now provides a method for the generation of calmodulins with specific alterations for further studies of the relationship of structure to activity. Future studies using posttranslational modification, site-specific mutagenesis methods, and purified calmodulin binding proteins will hopefully clarify further the structural basis of the interaction of calmodulin with NAD kinase and other target proteins.

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