The 3-Å crystal structure of calmodulin indicates that it has a polarized tertiary arrangement in which calcium binding domains I and II are separated from domains III and IV by a long central helix consisting of residues 65–92. To investigate the functional significance of the central helix, mutated calmodulins were engineered with alterations in this region. Using oligonucleotide–primed site-directed mutagenesis, Thr–79 was converted to Pro–79 to generate CaMPM. CaMPM was further mutated by insertion of Pro–Ser between CaM–Thr–78 and Pro–Thr–79 to yield CaMMIM. Calmodulin, CaMPM, and CaMMIM were indistinguishable in their ability to activate calcineurin and Ca\(^{2+}\)-ATPase. All mutated calmodulins would also maximally activate cGMP-phosphodiesterase and myosin light chain kinase, however, the concentrations of CaMPM and CaMMIM necessary for half-maximal activation (\(K_{\text{act}}\)) were 2- and 9-fold greater, respectively, than CaM23. Conversion of the 2 Pro residues in CaMMIM to amino acids that predict restoration of normal calmodulin activity. To investigate the nature of the interaction between mutated calmodulins and target enzymes, synthetic peptides modeled after the calmodulin binding region of smooth and skeletal muscle myosin light chain kinase were prepared and used as inhibitors of calmodulin-dependent cGMP-phosphodiesterase. The data suggest that the different kinetics of activation of myosin light chain kinase by CaM23 and CaMMIM are not due to differences in the ability of the activators to bind to the calmodulin binding site of this enzyme. These observations are consistent with a model in which the length but not composition of the central helix is more important for the activation of certain enzymes. The data also support the hypothesis that calmodulin contains multiple sites for protein–protein interaction that are differentially recognized by its multiple target proteins.

Calmodulin (CaM)\(^1\) is a member of a growing family of calcium binding proteins which includes troponin C, parvalbumin, and calbindins. Some of these calcium binding proteins, such as CaM and troponin C, serve as transducers of calcium signals. Both of these proteins modulate the function or activity of target proteins via calcium-dependent alterations in protein–protein interactions. At the molecular level, information required for transduction of the calcium signal is encoded by the spatial arrangement and dynamic properties of complementary recognition domains in the calcium-binding protein and its target protein.

The analogous mechanism of action of CaM and troponin C may have provided the selective pressure to maintain the very similar tertiary structure shared by these homologous proteins (1–3). Each protein consists of four calcium-binding domains that conform to the helix-loop-helix or EF-hand motif originally observed in parvalbumin (4). In both proteins, calcium binding domains I and II are separated from domains III and IV by a long \(\alpha\)-helix located in the central region of the proteins. Two other calcium binding proteins with known crystal structures, parvalbumin and the 7.5-kDa form of calbindin, also contain EF-hand calcium binding domains but do not have the elongated dumbbell shape of CaM and troponin C (4, 5). This suggests a basic difference in the mechanisms of action of these two types of calcium binding proteins. Indeed, parvalbumin and the 7.5-kDa calbindin have not been demonstrated to have activator activity. The similar tertiary motif of CaM and troponin C may be typical of a class of calcium “switch” proteins in which the specificity of the switch is defined by variations in protein recognition domains within this general structural framework. For example, the amino-terminal helix in troponin C, which is absent in CaM, may contribute to functional divergence.

The structure of CaM and how it encodes functional information is of particular interest since CaM regulates a variety of proteins and enzymes. A series of studies using biochemical (6–9) and protein engineering (10–13) techniques have shown that CaM contains multiple functional domains that selectively interact with target enzymes. In this report we have mutated the central helix of CaM to investigate its role as a structural element and a protein–protein interactive site and show that its length and not composition appears more important for the activation of selected enzymes. The data are also consistent with our previous classification of CaM-dependent enzymes based on their interactions with bacterially synthesized CaM-like proteins (10, 11).

**MATERIALS AND METHODS**

**Plasmid Construction—**Mutation of CaM was accomplished using oligonucleotide–primed site-directed mutagenesis of the CaM expression plasmid pCaM23 (10) as outlined in Fig. 1. In step 1 a 274-base pair EcoRI–PstI fragment from pCaM23 was subcloned into the bacteriophage M13mp18 to obtain a single stranded DNA template molecule, pTemplate-1. In step 2, primer 1 was used to convert Ser–79 to Pro and also generate a BamHI for screening and as a site for

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were heated to 55 °C for 10 min and cooled slowly to room temperature in 10 with 10 μl of 20 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 0.8 mM dNTPs, 1 unit of Klenow fragment DNA, 10 units of T4 DNA ligase. The reaction was incubated at 14-15 °C for 5 h, after which 1 μl was used to transform JM103 cells by the procedure of Hanahan (15). To screen for the desired mutation, step 3, the trans-
which 1 ml was used to transform JM103 cells by the procedure of

were subcloned into pCaM23 to yield pintermediate-1. To complete the amino acid coding region of pCaMPM a 700-base pair PstI fragment from pCaM23 was subcloned into the unique PstI of pintermediate-1 to yield pCaMPM.

A CaM insert mutation was generated using cassette mutagenesis of pCaMPM as outlined in steps 5 and 6 of Fig. 1. pCaMPM, which has two BamHI sites, was partially digested with BamHI, dephosphorylated with bacterial alkaline phosphatase, and ligated with a BamHI fragment from pUC4K (Pharmacia LKB Biotechnology Inc.) that contains kanamycin resistance markers within an inversion cassette. Ligation products were used to transform JM109, and kan-
amycin-resistant colonies were picked for restriction endonuclease analysis of their plasmid DNA. The desired plasmid, pintermediate-2, was digested with SalI, re-ligated, and used to transform JM103 cells. The resulting plasmid called pCaMIM is identical to pCaMPM with the exception of an additional 12 nucleotides that encode 4 amino acids between Asp-78 and Pro-79 of CaMPM.

Steps 8-12 of Fig. 1 outline procedures that convert the 2 Rd residues in CaMIM to helix-forming amino acids. An EcoRI/PstI fragment from pCaMIM was first subcloned into M13mp18 to yield template DNA for cassette-2. Mutated plasmid cassette-2 was subcloned in step 9 as described above using primer 2. In step 10, replicative form DNA was isolated from a mixed population of transformed bacteria, digested with BamHI, and used directly to transform JM103. Since replicative form DNA that have mutations in both Pro codons will be resistant to BamHI digestion, they will remain circular and have a much higher efficiency of transformation. Phage pbCaMIM-TQ, pbCaMIM-KQ, and pbCaMIM-QQ were identified first by restriction endonuclease analysis and then by DNA sequencing. In step 11 EcoRI/PstI fragments from the replicative form DNA of these three phage were subcloned into pCaM23 that had been digested with EcoRI and partially digested with PstI. In step 12, 4 coding region from pintermediates-3, 4 and 5 were isolated after digestion with EcoRI and partial digestion with PstI. These fragments were subcloned into pcCaMPL which had been digested with PstI and partially digested with EcoRI. pcCaMPL is a derivative of pcCaM23N (18) in which the tac promoter has been replaced with a heat-sensitive P₄ promoter (19).

**Protein Isolation and Enzyme Assays**—Bacterially synthesized CaMAs were isolated by phenyl-Sepharose chromatography as described previously (10, 18). As an additional purification step the protein was applied to a Weak阴 ion exchange liquid chromatography column and eluted with a NaCl gradient in a buffer of 50 mM Tris, pH 7.5, 0.2 mM EDTA. For those experiments where a change of buffer was required, the proteins were either desalted into the appropriate buffer by gel filtration using Sephadex G-25 or subjected to 6-8 successive rounds of concentration and desalting into the appropriate buffer by gel filtration using Sephadex G-25, or subjected to 6-8 successive rounds of concentration and desalting into the appropriate buffer by gel filtration using Sephadex G-25.

For cGMP-phosphodiesterase assays, CaM23 concentration was determined by amino acid analysis, and the concentration of CaM mutants was determined by the method of Bradford (20) using CaM23 as a standard. The concentration of CaM binding peptides was determined by absorption at 278 nm using molar extinction coefficients of 5556 and 5554 for skeletal and smooth muscle isoforms of myosin light-chain kinase, respectively.

Chicken myosin light chains and chicken light chain kinase were isolated as described previously (24, 25). Myosin light chains were chromatographed on phenyl-Sepharose CL-4B to reduce contaminations of CaM (either 1.7 mM CaM23; 3.4 mM CaMPM; or 16.5 mM CaMIM). 20% glycerol, and the phosphodiesterase reaction mixture as described above.

Chicken gizzard myosin light chains and myosin light chain kinase were isolated as described previously (24, 25). Myosin light chains were chromatographed on phenyl-Sepharose CL-4B to reduce contaminations of CaM (26). The assay was performed in a 0.1 ml volume containing 50 mM PEP, pH 8.0, 100 mM KCl, 1 mM MgCl₂, 1 mM DTT, 0.2 mM ATP (6-8 × 10⁻⁶ M of [γ-³²P]ATP), 0.05 mg/ml bovine serum albumin, 0.02 mM myosin light chains, 5 × 10⁻⁶ M myosin light chain kinase, and the indicated amount of activator. The mixture was incubated at 30 °C for 30 min. The reaction was terminated and incorporation of ³²P into myosin light chains was determined as described previously (27).

Calcineurin was isolated and assayed as described previously (6, 28). The assay was conducted as 28 °C and contained 20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM DTT, 5 mM p-nitrophenyl phosphate, 0.1 mg/ml bovine serum albumin, 0.1 mg/ml calcineurin, and the indicated amount of activator. Maximal stimulation of enzyme activity in the presence of saturating amounts of activator was 18-fold over basal activity in the presence of EGTA and 8-fold over the activity in the presence of Ca²⁺ but no activator. Rhodocyt e ATPase was isolated and assayed as described by Niggli et al. (29).
RESULTS

The amino acid changes introduced into CaM by the procedures outlined in Fig. 1 are summarized in Fig. 2, panel A. Bacterially synthesized CaM23 has the sequence of vertebrate CaM and is expressed from a plasmid containing the chicken calmodulin cDNA. Despite the absence of an acetylated amino terminus and trimethylation of Lys-115, CaM23 has been shown to be physically and functionally identical to naturally occurring calmodulin by all criteria tested thus far (10, 11) and is used as a control in this study. CaMPM contains a single point mutation in which Thr-79 is changed to a Pro residue. CaMIM is a derivative of CaMPM and has Pro-Ser-Thr-Asp inserted between Asp-78 and Pro-79. CaMIM-T (Thr), CaMIM-TQ (Thr and Gln), and CaMIM-KQ (Lys and Gln) are all derivatives of CaMIM which in one or both Pro residues are converted to the indicated amino acids. Multiple substitution of the Pro residues was accomplished using a mixture of oligonucleotide primers. The oligonucleotide was designed to replace the second Pro with Thr, Gln or Lys; however, only Gln was obtained. This probably reflects a more stable hybridization between the template and those oligonucleotides that encode Gln at the second Pro position. Although necessarily imprecise, secondary structure in the mutant proteins was predicted by the method of Garnier et al. (31) and is summarized in Fig. 2, panel B. This calculation assigns a numerical value for the probability that a given amino acid will be in either a random coil (C), turn (T), β-sheet (S), or α-helix (H) secondary structure. The program accurately predicts the non-helical regions of CaM23 which constitute the four Ca2+-binding loops and the non-helical region that connects domains I and II. The central region in CaM23 is assigned a helical configuration; however, the numerical value for Thr-79 indicates that this helix is not strongly favored. Analysis of the central region of CaMIM indicates that Lys-77 through Asp-80, including the 4-amino acid insertion, probably assume a non-helical conformation in the protein. Conversion of the Pro residues to either polar or charged amino acids predicts a more mild disruption of the α-helix with CaMIM-KQ predicted to retain an α-helical secondary structure across the insertion mutation. Similar to

A.

B.

Fig. 1. Construction of calmodulin expression plasmids. Steps 1-13 (panel A) are described under “Materials and Methods.” Terms that are prefaced with p are plasmid vectors. Terms that are prefaced with ph designate phage vectors. Primers used for site-directed mutagenesis are shown in panel B.

cDNA Primer 1 5'-GAAAATGAAAGATAGCTGATAGCGG-3' 3'-CTTTTACTTCGACTATCGG-5'
cDNA Primer 2 5'-ATGAAAGATTCGTCGACGGGTCC-3' 3'-TACTTCTTCAGGCTGTGCTGAGGGTCC-5'
Amino acid sequence of mutated calmodulins (panel A) and predicted secondary structure (panel B). Amino acids that differ from CaM23 are underlined in CaMIM-T, CaMIM-TQ, and CaMIM-KQ are those which differ from CaMIM. Regions of predicted secondary structure in the mutated CaM proteins are related in bar graph format. Each amino acid is assigned as participating in coil, turn, sheet, or helix configurations which are abbreviated as C, T, S, and H, respectively, on the left side of the diagram next to the names of the proteins. The roman numerals show the location of Ca++ binding loops.

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CaM23, helical conformation of the central region in CaMIM-KQ is not strongly favored.

The purity and electrophoretic mobility of the mutant CaMs is shown in Fig. 3. All proteins exhibited an equivalent Ca++-dependent decrease in apparent molecular weight. Proteins with insert mutations migrated slightly slower than CaM23 and CaMPM in the presence and absence of CaC12, consistent with an additional 4 amino acids. CaMIM-T, CaMIM-TQ, and CaMIM-KQ all co-migrated with CaMIM in the presence and absence of calcium (data not shown).

In an effort to detect potential differences in secondary structure, calcium-dependent changes in tyrosine fluorescence and CD spectra were compared for CaM23, CaMPM, and CaMIM. Fig. 4 shows that intrinsic tyrosine fluorescence is not affected by the amino acid changes in the central helix of CaM. This was not unexpected since the 2 tyrosine residues in CaM are located in domains III and IV at positions 99 and 138.

Fig. 5, panels A and B, show the CD spectra of CaM23 and CaMPM to be indistinguishable in the presence and absence of calcium. Panels C and D show the magnitude of the minima in the spectrum for CaMIM is about 10% greater relative to CaM23. This suggests that the sum of secondary structures between these two proteins is different but does not identify the nature of the difference. A recent paper by Hennessy et al. (33) suggests that, at high ionic strength, calcium-dependent changes in the secondary structure of CaM are due to a reorientation of helices rather than an increase in helical content. The differences in spectra between CaM23 and CaMIM may reflect general differences in the organization of secondary structures.

Fig. 6 shows the activation of calcineurin and Ca++-ATPase by CaM23, CaMPM, and CaMIM. Activation characteristics of both enzymes were unaffected by mutations in the central helix of CaM. In contrast, Fig. 7 shows that the activation characteristics of cGMP-phosphodiesterase and myosin light chain kinase are both influenced by alteration in the central helix of CaM. A summary of multiple experiments with cGMP-phosphodiesterase and myosin light chain kinase is shown in Table I. Although both enzymes are maximally stimulated by all bacterially synthesized CaMs, the concentrations of CaMPM and CaMIM necessary for half-maximal activation (Km) are approximately 2- and 9-fold greater, respectively, than CaM23. Although the apparent Km values from two experiments with myosin light chain kinase differ, the relative values shown in brackets in Table I are very similar. Inter-assay variability is most likely due to effects of storage on enzyme and substrate.

The aberrant ability of CaMIM to activate cGMP phosphodiesterase and myosin light chain kinase could be due to a disruption of secondary structure or a lengthening of the central region in CaM by 4 amino acids. To approach this...
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Fig. 5. Calcium-dependent CD measurements for CaM23, CaMPM, and CaMIM. Each panel shows the CD spectrum of CaM23 and either CaMPM or CaMIM. Panels A and B show CaM23 and CaMPM in the presence and absence of calcium, respectively. Panels C and D show CaM23 and CaMIM in the presence and absence of calcium, respectively. mdeg, millidegree.

Fig. 6. Activation of calcineurin (panel A) and erythrocyte ATPase (panel B) by CaM23, CaMPM, and CaMIM. Basal activity is the activity in the presence of calcium but not activator. Maximal activity is that obtained in the presence of calcium and saturating concentrations of CaM23.

Fig. 7. Activation of cGMP-phosphodiesterase (panel A) and myosin light chain kinase (panel B) by CaM23, CaMPM, and CaMIM. Basal activity of cGMP-phosphodiesterase is activity in the presence of calcium but without activator. Maximal activity is defined as activity in the presence of calcium and saturating concentrations of CaM23.
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TABLE I
Activation constants for activation of cGMP-phosphodiesterase and myosin light chain kinase by CaM23, CaMPM, and CaMIM

| Phosphodiesterase | Myosin light chain kinase |
|-------------------|--------------------------|
|                   | Experiment 1 | Experiment 2 |
| CaM23             | 1.2 ± 0.3 (20) | 0.45 ± 0.05 | 0.24 ± 0.40 |
| CaMPM             | 2.6 ± 0.5 (6) | 0.73 ± 0.10 (1.6) | 0.39 ± 0.11 (1.6) |
| CaMIM             | 9.3 ± 1.1 (5) | 4.18 ± 0.20 (9.3) | 2.75 ± 0.60 (8.6) |

The following numbers represent the apparent Kact (nanomolar) for enzyme activation by the indicated CaM. Kact is defined as the amount of CaM required for half-maximal activation under standard assay conditions. The values for phosphodiesterase are the average ± S.D. of n determinations (in parentheses). The values for myosin light chain kinase represent two separate experiments and the error values are derived from computer fits of the data. The numbers in brackets are the -fold increase in Kact for CaMPM and CaMIM relative to CaM23.

![Activation of cGMP-phosphodiesterase by CaMIM, CaMIM-T, CaMIM-TQ, and CaMIM-KQ.](image1)

![Inhibition of cGMP-phosphodiesterase activity by the CaM-binding peptide (SmK) from smooth muscle myosin light chain kinase.](image2)

**DISCUSSION**

A long central helix has been observed in both CaM and fast skeletal muscle troponin C from structural analysis or the crystal structures (1–3). Although thermodynamic arguments do not favor the stability of an eight-turn α-helix that is exposed to solvent, experimental evidence not only support the existence of the central helix but also calcium-dependent conformational changes in this region. It is proposed that solvent-exposed helices in troponin C are stabilized by intrahelical electrostatic and salt-bridge interactions between the basic and acidic amino acid side chains (36). Vacuum-UV CD measurements in the presence of calcium predict a helical content for CaM that is in good agreement with the crystal structure (39), and they predict that binding of calcium by CaM at physiologic ionic strength induces a reorganization of the helices rather than a change in helical content. Fluorescence anisotropy measurements on the dityrosine derivative of CaM in the presence of high calcium concentrations suggest that protein has a length equivalent to that predicted by the crystal structure (37) and that in the absence of calcium the protein appears more compact and exhibits segmental motion.

Dynamic changes in the central helical region of CaM is an attractive model that would explain not only spectral data but also calcium-dependent differential accessibility of this region to both proteases (38) and acetic anhydride (39). Dynamic changes could involve a collapse of the amino- and carboxyl-terminal lobes to shield the central helix as might be predicted from the crystal structure of troponin C and/or...
an increase in the conformational flexibility of this region. These are attractive models; however, it must be appreciated that structural studies of isolated CaM may not fully represent its biologically active conformation when complexed with its target enzymes. For example, binding of melittin and peptides derived from myosin light chain kinase induce conformational changes in both halves of CaM as determined by NMR changes (40, 41) and binding of CaM to target proteins increases its affinity for Ca$^{2+}$ (42). This presents the possibility that interactions of CaM with target enzymes may stabilize conformations that do not predominate in solution. Crystalization of CaM may approximate interactions with target enzymes.

Assuming that the central helix in CaM does exist either in the Ca$^{2+}$-bound form of the isolated protein or when complexed with a target protein, at least two possible functions for this structure can be hypothesized. First, the helix may maintain a proper linear and/or rotational orientation between the amino-terminal and carboxyl-terminal lobes such that recognition sites in these regions can functionally interact with complementary sites in target proteins. Alternatively, the central helix may encode recognition sites that are rendered accessible to target enzymes by Ca$^{2+}$. We have attempted to investigate these mechanisms by generating a series of mutated proteins in which the length and composition of the central helix in CaM is altered.

CaMIM, CaMIM-T, CaMIM-TQ, and CaMIM-KQ all have an insertion in the central helix of 4 amino acids but with varying degrees of predicted disruption of the helix. All four mutants exhibit identical functional abnormalities with respect to activation of phosphodiesterase. Therefore, functional characteristics of these activators are not due to the Pro residues. Aberrant activation of myosin light chain kinase by CaMIM does not appear to result from a disruption of a recognition site due to insertion of amino acids since CaM-binding peptides derived from both the smooth and skeletal kinases have similar affinities for CaM and CaMIM. Therefore, the length and composition of the central helix appears more important for the activation of certain target enzymes. A similar conclusion was reached for skeletal muscle troponin C by Reinach and Karlsson (49) who showed that conversion of Gly-92 to Pro did not alter the properties of troponin C in a reconstituted actomyosin ATPase assay. A requirement for a specified length of the central helix in troponin C has yet to be investigated by mutagenesis.

Using an analogous approach, Craig et al. (13) have reported that conversion of Glu 82-84 in the central helix to lysines effectively inhibits the ability of the mutated CaM to activate myosin light chain kinase and plant NAD kinase while the activation of phosphodiesterase is minimally affected. Although the Ca$^{2+}$-dependent CD spectra of control and Lys-substituted CaM are quite different, suggesting that this considerable change in local charge density has distal effects on protein secondary structure, this acidic cluster may represent a recognition site that is more important for activation of the former two enzymes. If so, this recognition site does not appear to overlap Thr-79 since CaM-binding peptides from smooth and skeletal muscle myosin light chain kinase show the same relative affinity for CaM23 and CaMIM. Together, these results suggest that the central helix contributes to the functional characteristics of CaM by both providing sites of protein recognition and maintaining either a proper orientation or linear relationship between the lobes in CaM, and that the requirement for these structural features vary for different CaM-dependent enzymes.

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