PROTEINS: Structure, Function, and Genetics 6:70–85 (1989)

Computational and Site-Specific Mutagenesis Analyses of the Asymmetric Charge Distribution on Calmodulin

P.C. Weber,[1] T.J. Lukas,[2] T.A. Craig,[2] E. Wilson,[2] M.M. King,[3] A.P. Kwiatkowski,[3] and D.M. Watterson[2]

[1]Central Research and Development Department, E.I. du Pont de Nemours Co., Wilmington, Delaware 19880-0228, [2]Department of Pharmacology, Vanderbilt University, and Laboratory of Cellular and Molecular Physiology, Howard Hughes Medical Institute, Nashville, Tennessee 37232-0295, and [3]Department of Chemistry, Ohio State University, Columbus, Ohio 43210-1173.

ABSTRACT **Calmodulin's calculated electrostatic potential surface is asymmetrically distributed about the molecule. Concentrations of uncompensated negative charge are localized near certain α-helices and calcium-binding loops. Further calculations suggest that these charge features of calmodulin can be selectively perturbed by changing clusters of phylogenetically conserved acidic amino acids in helices to lysines. When these cluster charge reversals are actually produced by using cassette-based site-specific mutagenesis of residues 82–84 or 118–120, the resulting proteins differ in their interaction with two distinct calmodulin-dependent protein kinases, myosin light chain kinase and calmodulin-dependent protein kinase II. Each calmodulin mutant can be purified to apparent chemical homogeneity by an identical purification protocol that is based on conservation of its overall properties, including calcium binding. Although cluster charge reversals result in localized perturbations of the computed negative surface, single amino acid changes would not be expected to alter significantly the distribution of the negative surface because of the relatively high density of uncompensated negative charge in the region around residues 82–84 and 118–120. However, this does not preclude the possibility of single amino acid charge perturbations having a functional effect on the more intimate, catalytically active complex. The electrostatic surface of calmodulin described in this report may be a feature that would be altered only by cluster charge reversal mutations. Overall, the results suggest that the charge properties of calmodulin are one of several properties that are important for the efficient assembly of calmodulin–protein kinase signal transduction complexes in eukaryotic cells.**

Key words: Protein electrostatics, protein kinases, effector protein, calcium-binding protein, α-helix

INTRODUCTION

Calcium has many biological functions, including the ability to act as an intracellular messenger or signal transducer in eukaryotic cells (for a review, see ref. 1). In response to a variety of stimuli, the intracellular concentration of ionized calcium transiently increases, then is restored to basal level by several means. This transient rise in ionized calcium concentration is transduced into a biological response by the reversible interaction with a class of calcium-binding proteins, one of which is calmodulin. Calmodulin alone has no enzymatic function, but acts as an integral calcium binding subunit of a variety of enzymes (for recent reviews, see refs. 2–4). Many calmodulin-regulated enzymes lack detectable activity until calcium binds to calmodulin, which brings about an activation of enzyme activity.

Although an area of intense investigation, the detailed molecular basis of these modulated protein–protein interactions is not known. In order to understand how calmodulin is able to form selective functional complexes with such diverse enzymes in the eukaryotic cell, which contains other proteins that are calmodulin analogs,[5,6] an increased knowledge about the structural basis of complex formation with selected enzymes must be provided. Interactions between calmodulin and target enzymes, such as myosin light chain kinase (MLCK) and the type II calmodulin-dependent protein kinase (CaMPKII), include formation of multisubunit complexes (e.g., ref. 7) as well as catalytically important interactions that are modulated in response to calcium binding to calmodulin.[8–11] Based on a variety of site-specific mutagenesis,[8,12–19] proteolysis,[20,21] and chemical modification studies,[22–27] these interactions are likely to involve structurally overlapping sites on the calmodulin molecule. Studies[16,28–33] of calmodulin and various calmodulin-binding model

Received February 15, 1989; revision accepted June 19, 1989.

Address reprint requests to D. Martin Watterson, 823 Light Hall, Vanderbilt University, Nashville, TN 37232-0295.

A.P. Kwiatkowski's current address is 823 Light Hall, Vanderbilt University, Nashville, TN 37232-0295.

compounds have demonstrated the importance of hydrophobic interactions and charged amino acid residues in calmodulin–peptide or calmodulin–drug complex formation.

Consistent with results from peptide analog studies, investigations with holoenzymes suggest the involvement in some instances of ionic interactions in calmodulin-dependent enzyme activation. For example, the activation of myosin light chain kinase by calmodulin is attenuated at higher ionic strengths,[34] and calmodulin binding to immobilized calcineurin is slightly decreased by high ionic strength.[35] Although the interaction of myosin light chain kinase with its protein substrate also has an ionic component,[32,34] the importance of electrostatics in the interaction of calmodulin with myosin light chain kinase is supported by other studies. For example, site-specific mutagenesis of a Glu-Glu-Glu cluster at residues 82–84 in calmodulin to Lys-Lys-Lys selectively alters the interaction with myosin light chain kinase.[13,15] Overall, the data from peptide analog, site-specific mutagenesis and enzymological studies suggest an electrostatic component to calmodulin's interaction with macromolecules, including the multiple calmodulin-regulated protein kinases.

With the availability of a crystal structure[36,37] and a system for efficient site-specific mutagenesis of calmodulin at single or multiple sites,[38] one initial approach that might provide some insight into the role of electrostatics in calmodulin function is the combined use of computational techniques and site-directed mutagenesis in conjunction with functional assays. Although the available crystal structure of calmodulin is that of vertebrate calmodulin, the analysis of the primary structure and function of calmodulins from widely divergent phylogenetic species[2,38–41] and initial site-specific mutagenesis studies[13,14,15,17,38] suggest that this one structure may be a useful starting point in attempts to gain insight into how calmodulin structure is related to its function. Briefly, the amino acid sequences of calmodulins isolated from diverse phylogenetic species are invariant at 64 of the core 148 amino acids. The variation of calmodulin at the remaining 84 residues has little effect on calmodulin function with some enzymes, such as phosphodiesterase,[2,38,39,41] but does have a significant effect on calmodulin activity with other enzymes, such as NAD kinase.[12,38,39] A calmodulin that was designed to be fully functional with all calmodulin-regulated enzymes tested has been produced in *Escherichia coli,* an organism that lacks an endogeneous calmodulin, by the expression of a synthetic gene.[38] This calmodulin, termed VU-1 or SYNCAM calmodulin,[38] has an amino acid sequence that is a hybrid of the vertebrate[42,43] and plant[44] calmodulins. Thus, SYNCAM calmodulin represents a calmodulin with enhanced activity, compared to vertebrate calmodulin, for some enzymes (e.g., plant NAD kinase), but is an isofunctional analog of vertebrate calmodulin for other calmodulin-regulated enzymes (e.g., vertebrate MLCK). In this regard, SYNCAM calmodulin can be viewed as a fully active standard of comparison and provides a firm starting point for the functional aspect of studies that attempt to correlate calmodulin structure with function.

In the study described here, we have (1) constructed atomic models of SYNCAM calmodulin based on the α-carbon backbone of rat testis calmodulin,[36] (2) calculated the electrostatic properties of SYNCAM calmodulin, (3) identified regions with a relatively high electrostatic potential, (4) identified possible mutations of SYNCAM calmodulin that would alter these charge features, and (5) directly assessed the possible functional significance of these changes of calmodulin by using site-specific mutagenesis, protein purification, and enzyme activation analysis.

MATERIALS AND METHODS

General Procedures

Oligonucleotides were prepared on an Applied Biosystems DNA synthesizer and purified as described previously.[13,45] The general approach for mutagenesis and protein production is described in Roberts et al.[38,45] and Craig et al.[13] The presence of the desired mutations in the final cloned constructs was verified by automated DNA sequence analysis of the gene as described in Zimmer et al.[46] and restriction endonuclease mapping.[13] Proteins were produced in *E. coli,* purified, and characterized by amino acid compositional and amino terminal sequence analysis using previously described approaches.[12,38,47] Amino acid compositions of acid hydrolysates of protein and peptide samples were determined by using precolumn derivatization with phenylisothiocyanate and HPLC analysis following the general method of Bidlingmeyer et al.[48] with the modifications described in Schaefer et al.[47] Calmodulin activity in smooth muscle myosin light chain kinase assays was determined following the general procedures previously described[31]; the standard assay conditions were 50 mM Hepes, 5 mM Mg^{2+}, 0.1 mM Ca^{2+}, at pH 7.5, with an ATP + [γ-^{32}P]ATP concentration of 0.2 mM and DTT added to a final concentration of 1 mM. Calmodulin-dependent protein kinase II assays were done essentially as described[49] previously except the concentrations of enzyme (2 nM), [γ-^{32}P]ATP (100 μM), and a synthetic peptide substrate (5 μM) (PLRRTLSVAA) were changed. All assays were performed at 25°C unless stated otherwise. Calcium dependence under the conditions used in this report was screened for by the substitution of EGTA for calcium.

SYNCAM, SYNCAM-8, SYNCAM-12A, and SYNCAM-18A calmodulins were biosynthetically labeled with [^{35}S]sulfate to a similar specific activity,

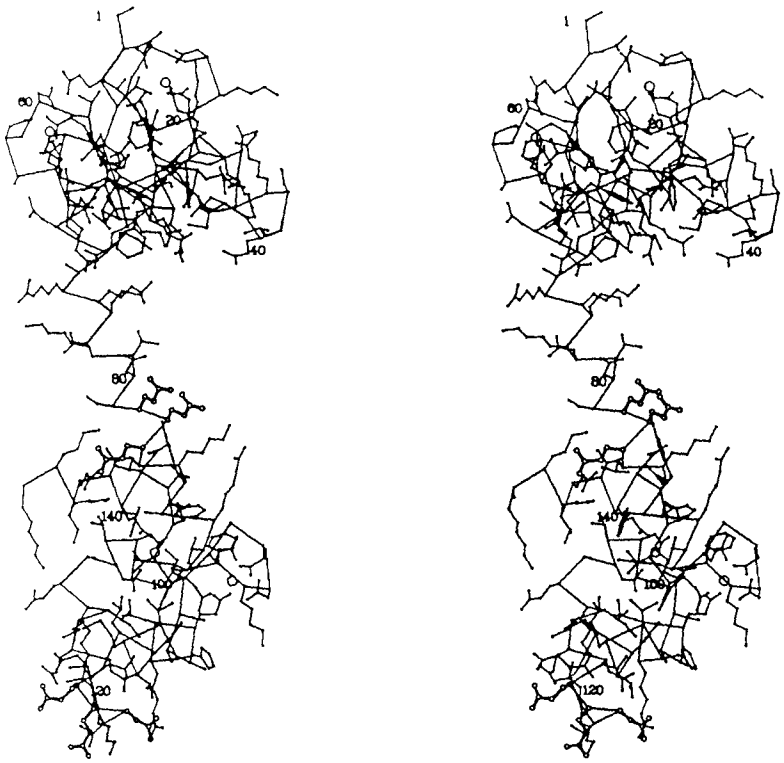


Fig. 1. Stereoviews of a three-dimensional model of SYNCAM calmodulin. The SYNCAM calmodulin was generated by the model-building procedure described in the text using the α-carbon trace of a vertebrate calmodulin as the starting structure. For clarity, the polypeptide backbone is shown as lines interconnecting α-carbon positions. All side chain atoms are included. Large open circles indicate bound calcium positions. The amino-terminus and selected α-carbons are labeled. Positions of acidic side chains changed to lysines in the various mutants are highlighted by open circles interconnected by wider bonds.

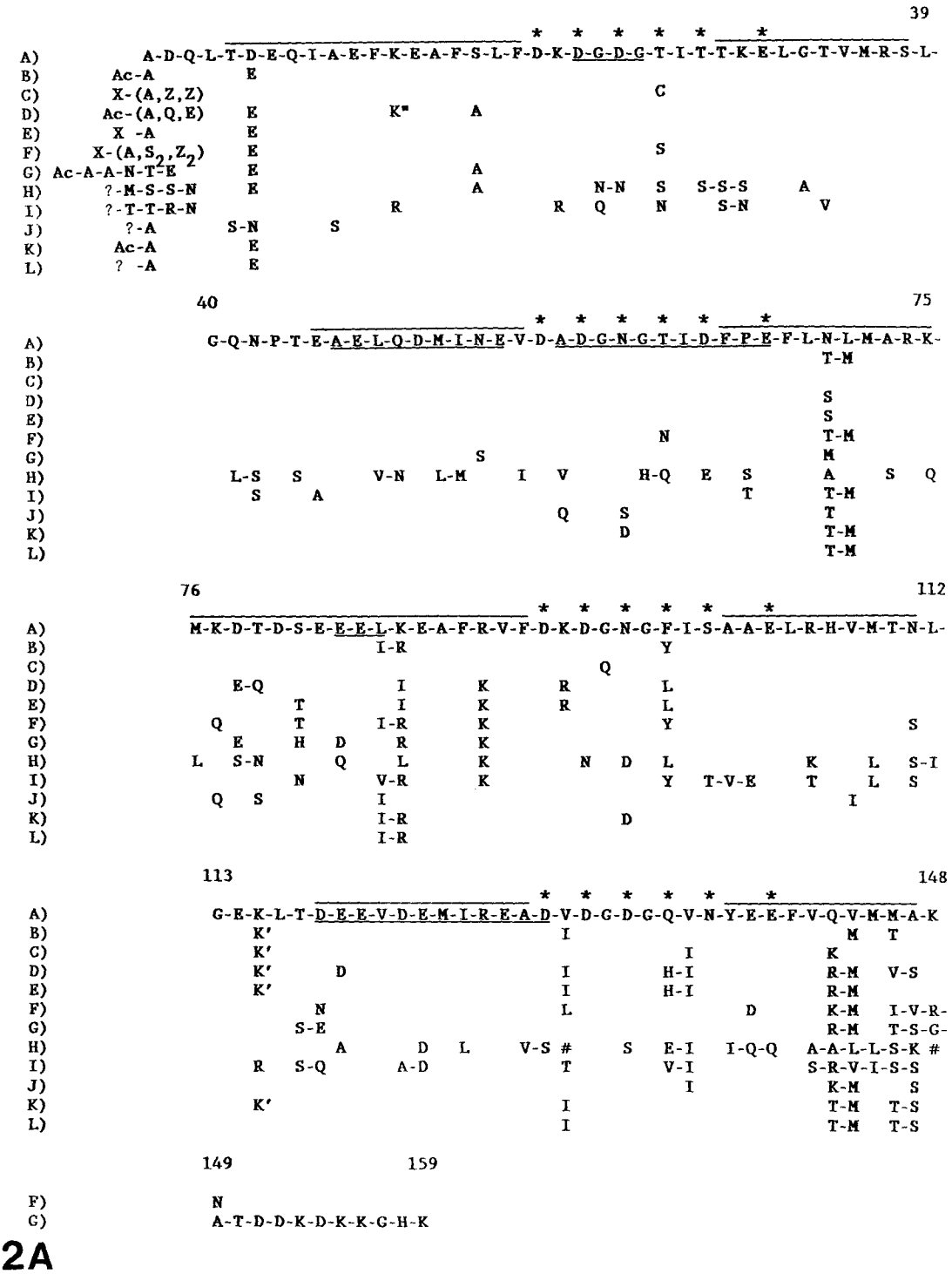
purified, and used as ligands in a gel overlay procedure essentially as previously described.[50,51] Briefly, the enzyme samples were subjected to SDS-PAGE, the gels were fixed in isopropanol:acetic acid:water (5:2:13, v/v/v), and in situ renaturation was done in 50 mM Hepes, 5 mM Mg^{2+}, and 150 mM NaCl, pH 7.5. Biosynthetically labeled calmodulins were added to each gel in 100 ml of buffer (50 mM Hepes, 5 mM Mg^{2+}, 150 mM NaCl, 0.1 mM $CaCl_2$, pH 7.5). Control incubations were done under identical conditions except for the substitution of 0.1 mM EGTA for $CaCl_2$ in the buffer. After incubation, the gels were stained with Coomassie Blue R250, destained, and dried. The dried gels were subjected to autoradiography using Kodak X-Omat X-ray film.

Model Building and Computational Studies

A model building procedure that uses α-carbon coordinates of a known structure to build a complete atomic model of a related structure was used to generate coordinates for SYNCAM calmodulin from rat testes calmodulin. The α-carbon coordinates of rat testes calmodulin[36] obtained from the Brookhaven Protein Data Bank[52] (Release 39, 1/87) served as the starting model for SYNCAM calmodulin. Rat testes and SYNCAM calmodulin are identical at 137 of 148 residues,[38] a level of sequence similarity that justifies using the rat testes structure as a starting model for SYNCAM calmodulin.

In the model building procedure,[53] the complete backbone is first generated from the α-carbon coordinates. Five to seven consecutive α-carbons of the known structure are used as a template to search a protein structural data base for similar structural segments. When a match between template and structure in the data base is found, backbone atoms from that structure in the data base are incorporated into the model of the related protein. The modeled protein, SYNCAM calmodulin, was sequentially built from the amino- to carboxy-terminus using α-carbon templates that overlapped by one or two residues. Generation of complete backbone coordinates by this method depends on the observation that regular secondary features of proteins exhibit a high degree of structural similarity.[53] In this work, the structural data base was composed of 48 highly refined crystallographic structures obtained from the Brookhaven Protein Data Bank.[52]

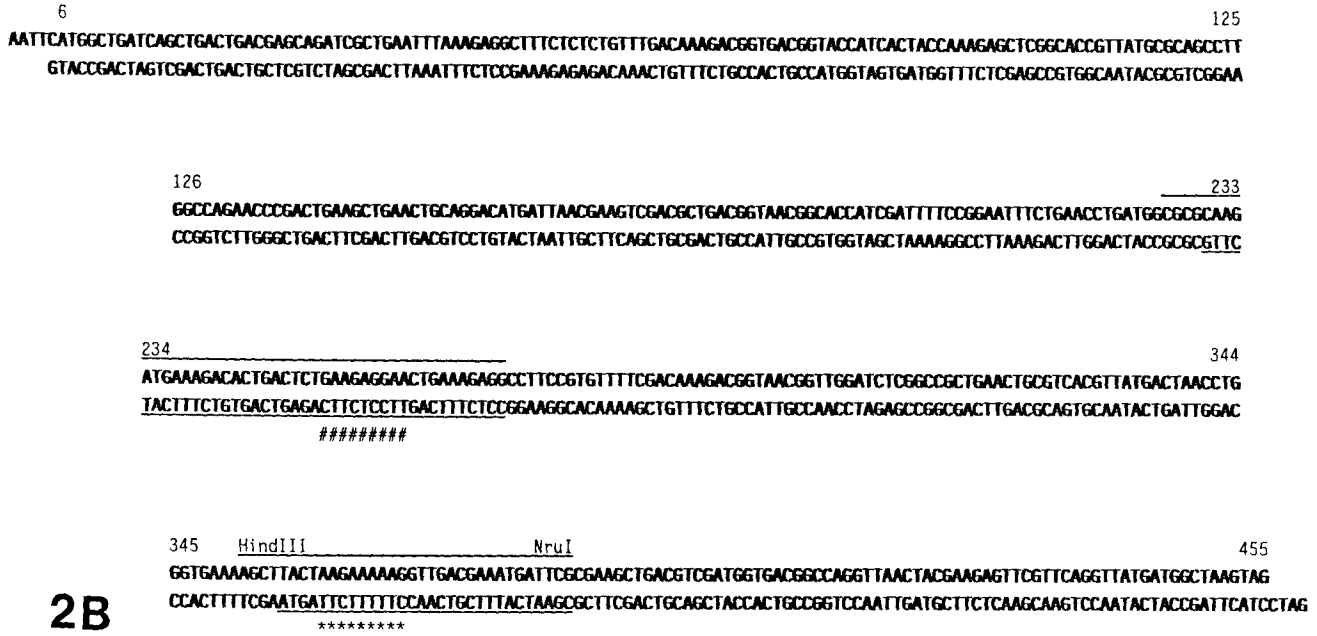
Side chain atoms were sequentially added to the SYNCAM model after all backbone atoms were positioned. The residue whose side chain was about to be added was placed at the center of a 5- to 11-



2A

Fig. 2A. Legend on pg. 74.

residue template. Side chain conformations of the central residue were tabulated for each match of the template within the structural data base. As a result, the statistically most probable conformation for each side chain within the context of secondary structure defined by the search template was determined and incorporated into the model. Frequently only one of the preferred rotamers[54] for an individual side chain was significantly statistically favored. Atomic positions were checked for van der Walls overlap and the energy of the model was minimized by several cycles of steepest descent and



2B

Fig. 2. Nucleotide and amino acid sequences for calmodulins. The amino acid sequences of synthetic (SYNCAM) and naturally occurring calmodulins are shown in **A**, and the DNA sequence for the SYNCAM-12A calmodulin gene is shown, along with differences from those of other SYNCAM calmodulins, in **B**. (**A**) The amino acid sequence of SYNCAM calmodulin is shown on the first line. Only those amino acids that differ from SYNCAM are shown for all other sequences. The asterisks denote residues or backbone positions involved in calcium binding and overlined residues correspond to α-helical regions determined from X-ray crystallographic studies of vertebrate calmodulin.[37] Underlining of residues indicates residues around which localized regions of comparatively high electrostatic potential are found (see Fig. 3). Amino-terminal blocking groups are represented by Ac- where an acetyl group has been verified or X where an unknown amino-terminus blocking group is present. A question mark, ?, is used to denote that the state of the amino-terminus is unknown when the sequence is derived from translation of a cDNA. Posttranslational modifications trimethyllysine and dimethyllysine are represented by K′ and K″, respectively. The symbol # represents a gap in sequence inserted for optimal sequence alignment. The other sequences shown are (B) bovine brain calmodulin[42,43]; (C) spinach calmodulin[44]; (D) *Paramecium* calmodulin[47]; (E) *Tetrahymena* calmodulin (Watterson et al., in preparation); (F) *Dictyostelium* calmodulin[41]; (G) *Chlamydomonas* calmodulin[46,84]; (H) yeast calmodulin—*Saccharomyces cerevisiae*[80]; (I) yeast calmodulin–*Schizosaccharomyces Pombe*[81]; (J) *Trypanosoma* calmodulin[85]; (K) scallop muscle calmodulin[86]; and (L) *Drosophila melanogaster* calmodulin.[87] (**B**) The double-stranded nucleotide sequence for the coding region of the SYNCAM-12A calmodulin gene is shown. The DNA sequence of the final construct was verified by using automated DNA sequence analysis. Protein coding begins with nucleotide 6 and the initial methionine is cleaved to produce the mature protein.[38] The cassette region inserted into the SYNCAM calmodulin gene in order to make the SYNCAM-12A gene is indicated by over- and underlining of nucleotides 352–385. The * below a nucleotide denotes that the nucleotide is different from that found in the SYNCAM gene.[38] The over- and underlining of nucleotides 226–270 indicate the region where the SYNCAM-8 cassette was inserted into the SYNCAM calmodulin gene.[13] The # symbol represents nucleotides that differ in SYNCAM-8 from the SYNCAM gene. Both of these cassette regions were used in the construction of the SYNCAM-18A gene. Thus, the amino acid sequences of SYNCAM-8, SYNCAM-12A, and SYNCAM-18A differ from that of SYNCAM shown in **A** by the presence of lysines at, respectively, residues 82–84, residues 118–120, or residues 82–84 and 118–120.

conjugate gradient minimization.[55] Side chain changes for the mutant proteins were built into the SYNCAM calmodulin model by the same conformational search procedure. Models of SYNCAM-8, -12A, and -18A calmodulins were energy minimized to the same convergence criteria used for the SYNCAM calmodulin model.

Electrostatic properties of SYNCAM and the mutant calmodulins were calculated using a modified Tanford–Kirkwood algorithm where the contribution of each charged atom is weighted by its solvent accessibility.[56,57] The effective charge for each titratable group in the protein is computed as a function of neighboring charges in the protein structure and solution parameters such as pH and ionic strength. In the computations, the four calcium ions of calmodulin were fixed as doubly charged cations. The stability of electrostatic potentials to picosecond protein dynamics simulations has been recently demonstrated in two independent systems.[58,59]

Production of SYNCAM-12A Calmodulin

A 30 base pair DNA cassette was prepared from the oligonucleotides shown in Figure 2B. The cassette contained codons for three lysines in place of the three acidic residues at positions 118–120 (see Fig. 2A). A new *Dde*I site was created by using, in the cassette, the lysine codon AAG at nucleotides 360–362. This new site facilitated screening of recombinant clones for the presence of the cassette. The cassette was phosphorylated and ligated with the appropriately cut pVUCH-1 vector.[60] The

TABLE I. Calculated Electrostatic Parameters for SYNCAM Calmodulins*

Calmodulin	pH	Ionic strength	Overall charge	Electrostatic free energy of stabilization (kcal/mol)	Difference of stabilization relative to SYNCAM (kcal/mol)
SYNCAM	7.0	0.01	−23.9	−46.4	0
SYNCAM	7.0	0.10	−24.0	−50.7	0
SYNCAM-8	7.0	0.01	−17.9	−47.5	−1.1
SYNCAM-8	7.0	0.10	−18.0	−50.5	+.2
SYNCAM-12A	7.0	0.01	−17.9	−45.9	+.5
SYNCAM-12A	7.0	0.10	−18.0	−48.7	+2.0
SYNCAM-18A	7.0	0.01	−12.0	−35.9	+10.5
SYNCAM-18A	7.0	0.10	−12.0	−33.8	+16.9

*Calculations were done with a simulated temperature of 298.16 K. The solvent accessible probe radius was 1.4 Å. The assumed dielectric constants were 78.5 in the solvent and 4.00 inside the protein.

ligation mixture was then used to transform competent *E. coli* UT481 cells. Randomly selected clones were screened for the presence of the mutant cassette by digestion of small-scale plasmid preps with the restriction enzyme *Dde*I and separation of DNA fragments by polyacrylamide gel electrophoresis. Clones positive for the presence of the SYNCAM-12A calmodulin gene by *Dde*I restriction mapping were characterized further by DNA sequence analysis and a construct with an experimentally verified correct DNA sequence was used for protein production.

Production of SYNCAM-18A Calmodulin

The approach used in the design and production of SYNCAM-18A calmodulin was to introduce the cassette shown in Figure 2B, that codes for the change in SYNCAM-12A calmodulin, into a vector/calmodulin gene construct containing the SYNCAM-8 calmodulin mutation. Briefly, the pVUCH/calmodulin gene construct[60] was cleaved with *Hin*dIII and *Nru*I, dephosphorylated, and ligated with the phosphorylated 30 base pair cassette shown in Figure 2B. Small-scale plasmid preps were screened for the presence of two additional *Dde*I sites compared to pVUCH-1.

As with the other constructions, clones positive by restriction mapping were characterized further by automated DNA sequence analysis and the correct construct used for protein production.

RESULTS

Model of SYNCAM Calmodulin and Computation of Electrostatic Properties

The model building procedure preserved the overall features of vertebrate calmodulin in the SYNCAM calmodulin model (Fig. 1, Materials and Methods). In a direct comparison of the SYNCAM calmodulin model and that based on the diffraction data for vertebrate calmodulin, the root mean square difference between backbone atoms of SYNCAM calmodulin and the crystallographically refined calmodulin (3 CLN entry, Brookhaven Protein Data Bank,[52] release 44, July 1988) was less than 0.9 Å. Like the vertebrate calmodulin structure,[37] SYNCAM calmodulin contains sets of structurally similar domains situated at opposite ends of an α-helix. Two calcium ions bind at structurally similar sites within each domain half of the molecule. The calculated net molecular charge for 14 positive and 37 negative amino acids (Fig. 2), other titratable groups, and the four calcium ions of SYNCAM calmodulin is −24.0. Despite this excess negative charge, the calculated electrostatic free energy of stabilization for this molecule is −50.7 kcal/mol (Table I). Models of other calmodulins were constructed (Materials and Methods) and their electrostatic parameters are also given in Table I.

Calmodulins from a diverse set of phylogenetic species have been characterized and comparisons of selected primary structures to SYNCAM calmodulin are shown in Figure 2A, with annotations that relate the structure shown in Figure 1 to the amino acid sequence. The amino acid sequence of calmodulin has four similar sequences, each of which includes a structural motif of helix–calcium ligation sequence–helix. Although there is some variation in the length of calmodulin sequences, there are 64 invariant residues that are found throughout the molecule. Many of these phylogenetically invariant residues are acidic amino acids in α-helices. As discussed below, some of the phylogenetically conserved segments of calmodulin appear to be coincident with some of the localized concentrations of uncompensated charge.

The excess negative molecular charge of calmodulin is asymmetrically distributed over the calmodulin molecular surface. Figure 3 shows the electrostatic potential surface for SYNCAM calmodulin calculated for pH 7.0 and a 0.15 M solution ionic strength. Under these conditions, the electrostatic potential surface is almost entirely negative (Fig. 3A). The negative potential surface is located predominately on one side of the molecule (Fig. 3B). This asymmetric distribution of charge is especially apparent when the electrostatic potential surfaces are viewed parallel to the long molecular axis (Fig.

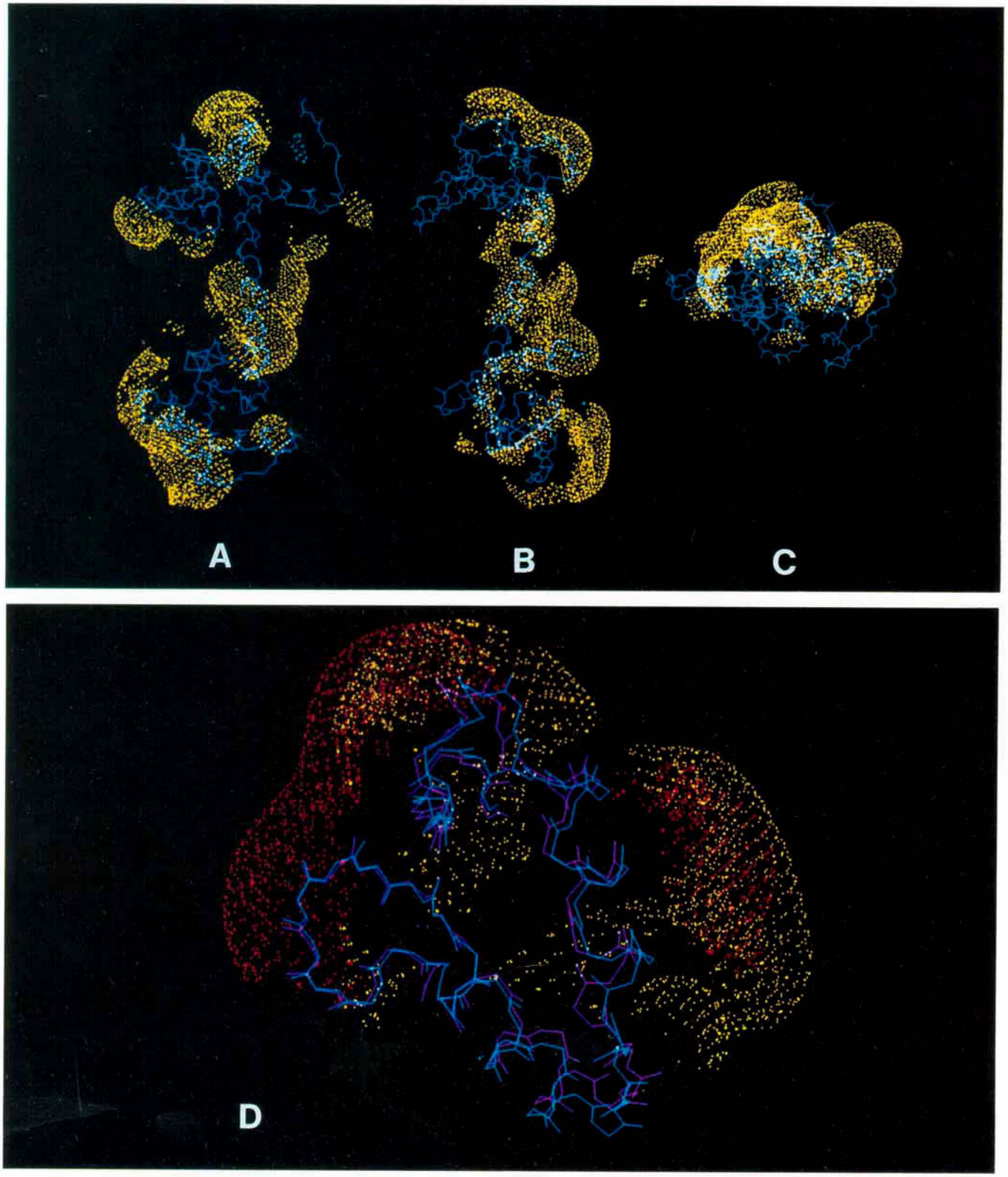


Fig. 3. Molecular symmetry and electrostatic surface potentials of calmodulin. Like vertebrate calmodulin,[37] SYNCAM calmodulin shows structural symmetry about the central helix. The ability of an electrostatic potential surface to introduce asymmetrical features is shown in **A–D.** Two displays of the calculated electrostatic potential surface of SYNCAM calmodulin (pH 7.0, $\mu \simeq 0.15$) are shown on a vertical (**A** and **B**) and horizontal axis (**C**). The potentials shown are the −1.0kT (yellow dots) and +1.0kT (blue dots) contours. The views shown in **A** and **B** have the molecule oriented such that the carboxy-terminal domain is at the bottom and the amino-terminal domain is at the top. The view in **C** is from the amino-terminus projected on an axis perpendicular to the page. **D** illustrates a superimposition of the top and bottom half of the molecule. The amino-terminal (−1.0kT) surface is in red and the carboxy-terminal surface is in yellow. Although the α-carbon traces for the two halves of the molecule are almost superimposable in **D,** the electrostatic potential surfaces of the two halves (red vs. yellow) are not.

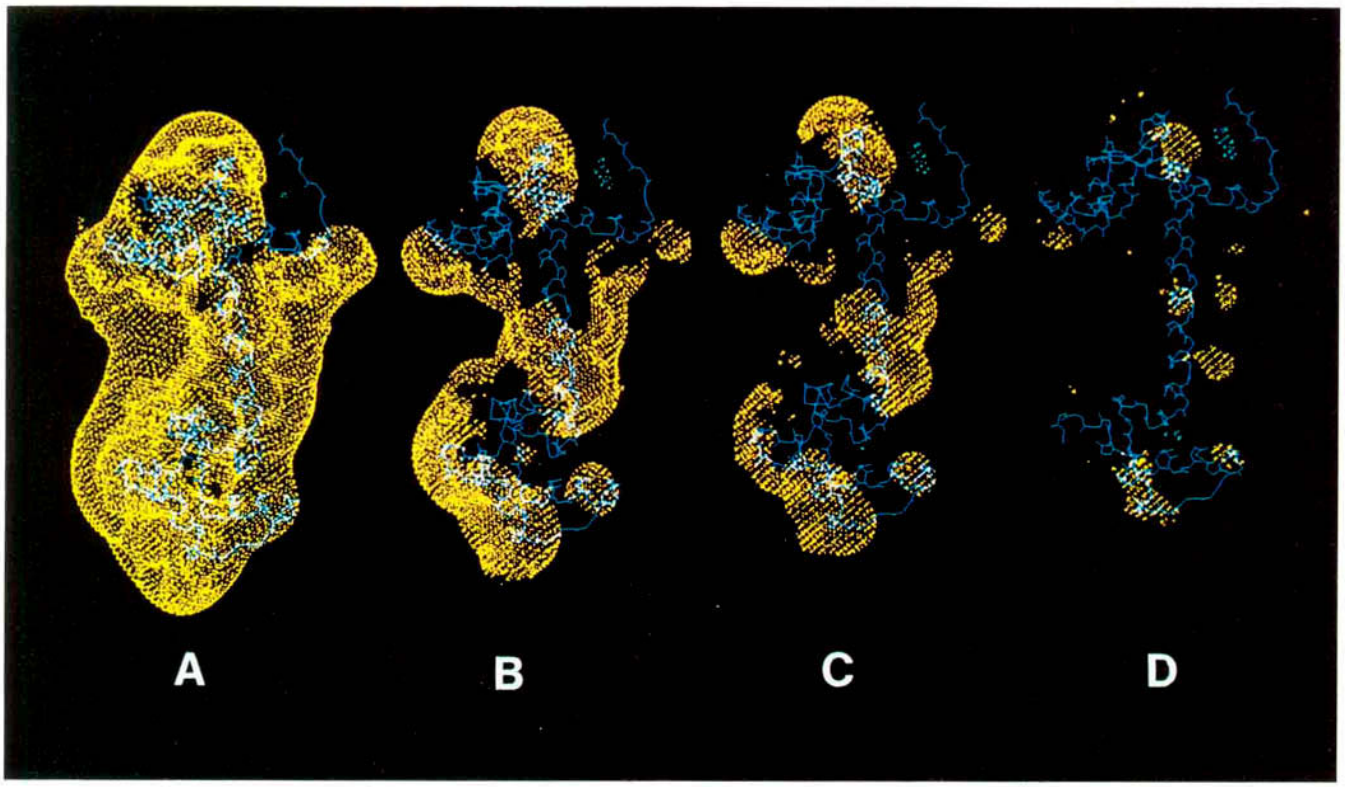


Fig. 4. The effect of ionic strength on the electrostatic surface potentials of SYNCAM calmodulin. The effects of ionic strength on electrostatic surface potentials were calculated by changing the ionic strength parameters within the algorithms described in Materials and Methods. Surfaces at pH 7.0 and ionic strengths of 0.04 (**A**), 0.1 (**B**), 0.15 (**C**), and 0.5 (**D**) are shown in this figure. Negative surfaces (−1.0kT) are indicated by yellow dots and positive surfaces are shown by blue dots (+1.0kT). The calmodulins are oriented with amino-terminus at the top of the figure.

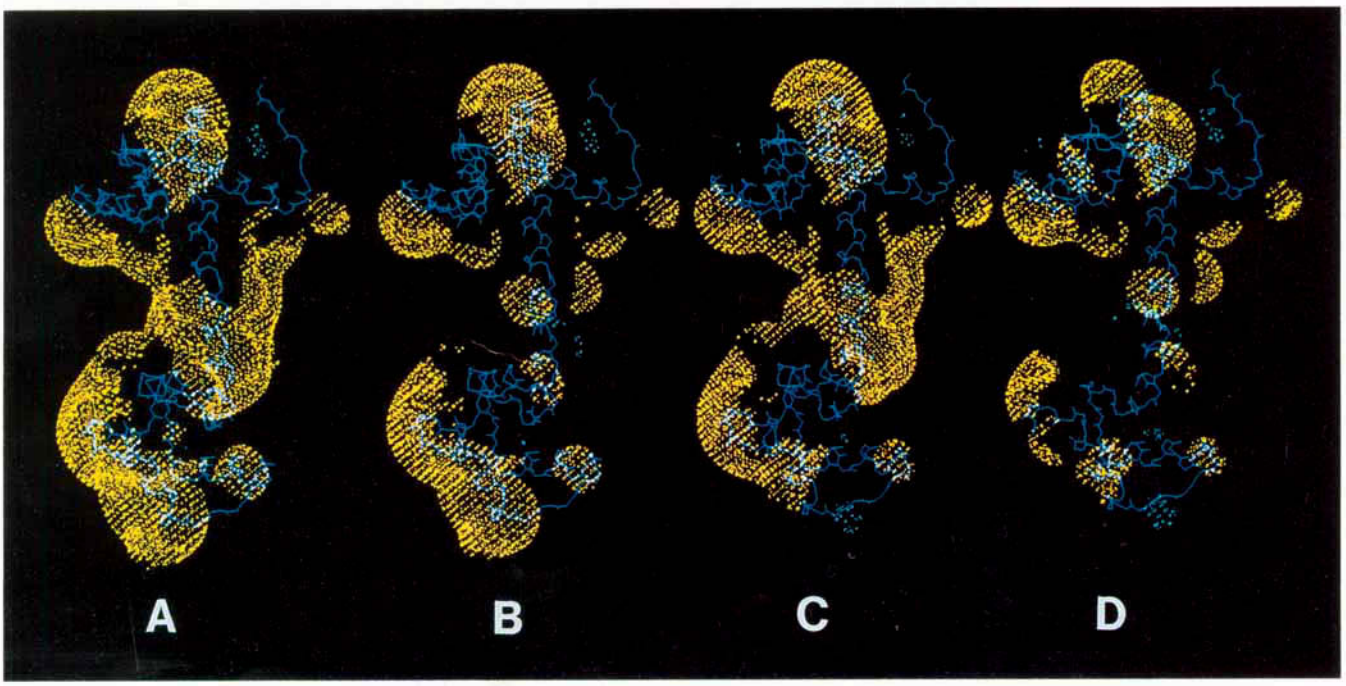


Fig. 5. Comparison of the electrostatic surfaces of SYNCAM and mutant calmodulins. Shown are the calculated surfaces for SYNCAM (**A**), SYNCAM-8 (**B**), SYNCAM-12A (**C**), and SYNCAM-18A (**D**) at pH 7.0 and ionic strength 0.1. The negative surfaces (-1.0kT contour) are shown by yellow dots, while the positive potential surfaces (+1.0kT) are shown by blue dots, with the molecule oriented such that the carboxy-terminal domain is toward the bottom. The relative positions of the side chains altered in each mutation can be located by noting the loss of yellow dots and the appearance of blue dots in each panel (central helix in **B**; bottom helix in **C**; both areas in **D**).

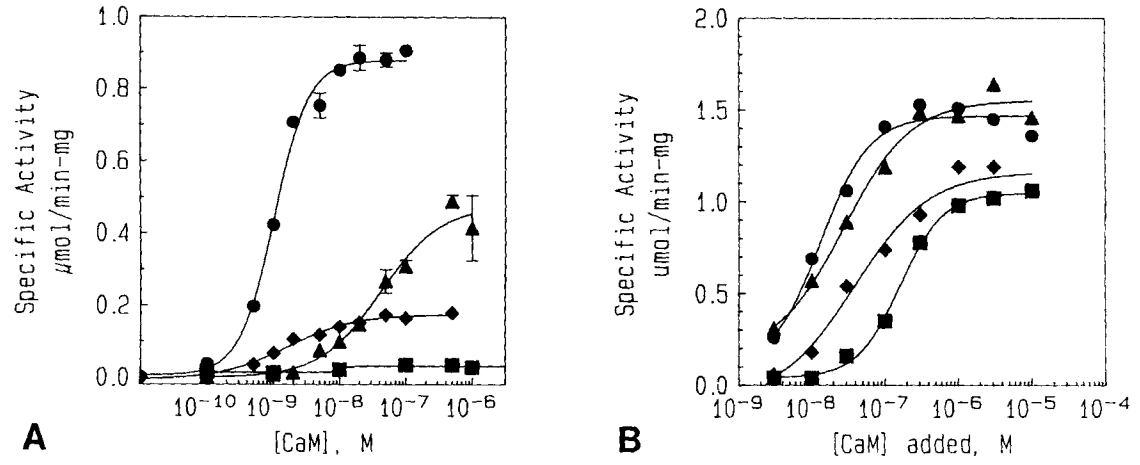


Fig. 6. Activation of protein kinases by calmodulins that differ in their computed electrostatic properties. **A** illustrates the ability of the fully active standard of comparison, SYNCAM calmodulin (●), and mutant calmodulins [SYNCAM-8 (▲), SYNCAM-12A (◆), and SYNCAM-18A (■) calmodulins] to activate vertebrate smooth muscle myosin light chain kinase phosphorylation of a synthetic peptide substrate (KKRPQRATSNVFAM) based on the light chain phosphorylation site. **B** shows the ability of the same calmodulins to activate calmodulin-dependent protein kinase II mediated phosphorylation of a synthetic peptide substrate (PLRRTLSVAA) based on a glycogen synthase phosphorylation site.

3C). Superposition of the structurally similar N- and C-terminal domains shows that each possesses a unique charge arrangement (Fig. 3D).

The extent of the SYNCAM calmodulin electrostatic potential surface depends on solution ionic strength (Fig. 4). At low (0.04 M) ionic strength, the surface nearly envelopes the entire molecule (Fig. 4A). The electrostatic potential surface shrinks and becomes more localized with increasing ionic strength (Fig. 4B,C,D). These results indicate the sensitivity of the electrostatic surface to ionic strength and highlight the localized concentration of uncompensated charge around phylogenetically conserved regions of the calmodulin amino acid sequence. For example, large continuous regions of negative electrostatic potential are located near residues 22–26 and 58–67 in the amino-terminal half of the molecule and residues 118–128 in the carboxy-terminal half. Smaller charge regions occur near residues 47–54 and around residues 114–115. Excess negative charge is also localized at the central helix near residues 83–85.

Investigation of the Possible Functional Significance of Charge Features, Central Helix Residues 82–84

Initial attempts to correlate these charge features of calmodulin with biological function included further computations on an existing charge reversal mutant (SYNCAM-8 calmodulin) in which the glutamic acid triad at residues 82–84 of the central helix was changed to three lysines.[13] Earlier studies[61] with synthetic peptides showed that glutamic acid and lysine residues at the same relative position in heteropolymers and homopolymers have similar helix-forming potential, and mutations of charged residues in α-helices have been shown to produce localized effects on the protein structure.[62,63] In addition, analysis of peptide analogs of the calmodulin-binding site[31] in MLCK suggested the importance of positively charged side chains in the interaction with calmodulin, a negatively charged protein.

The changes in negative electrostatic potential illustrated in Figure 5 for SYNCAM-8 calmodulin indicate that the effect of the cluster charge reversal mutation is relatively localized. As such, changes of any single negatively charged residue to a lysine might also be expected to exhibit a localized effect. The detection of such effects, however, is more difficult because of the compensation caused by other remaining negatively charged residues.

The slight increase in computed electrostatic stabilization free energy for the SYNCAM-8 calmodulin relative to SYNCAM calmodulin (Table I) may be due to insertion of positively charged residues within a negatively charged region of the molecule. Some of the apparent increase in stabilization energy may, therefore, be reflected in the central helix as a result of ion pairing. The preservation of the overall calmodulin structure in SYNCAM-8 calmodulin is indicated by several criteria: (1) SYNCAM-8 calmodulin was purified by a protocol identical to that used for the native calmodulin; this protocol is based on the physical properties of calmodulin and includes a calcium-dependent adsorption chromatography step; (2) although the activity with MLCK is diminished,[13] this reduced activity is calcium dependent; (3) the calcium-dependent activity with other calmodulin-dependent enzymes, e.g., phosphodiesterase[13] and calmodulin-dependent protein kinase II (Fig. 6), are minimally affected; (4) the

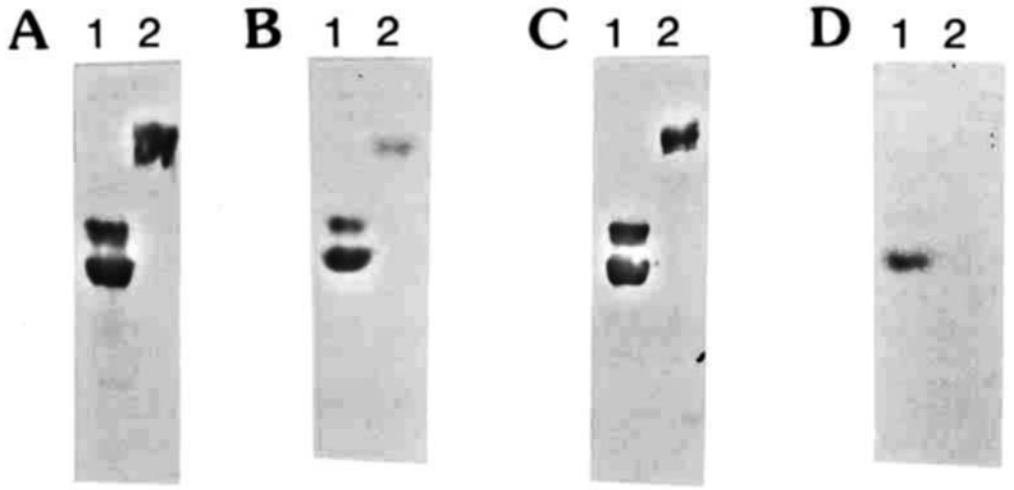


Fig. 7. Binding of metabolically labeled calmodulins to protein kinases in a gel overlay assay. The ability of purified SYNCAM (**A**), SYNCAM-8 (**B**), SYNCAM-12A (**C**), and SYNCAM-18A (**D**) calmodulins to bind to purified calmodulin-dependent protein kinase II or myosin light chain kinase in a gel overlay assay was determined as described under Materials and Methods. Calmodulin protein kinase II (Lane 1 in each panel; $M_r = 50{,}000$ and 60,000) is isolated as a dodecamer composed of the 50,000 M_r and the 60,000 M_r proteins in a ratio of 3:1, respectively. Myosin light chain kinase (Lane 2 in each panel; $M_r = 130{,}000$) exists as a heterodimer of calmodulin and a catalytic subunit (M_r = 130,000). The enzyme activation properties of these calmodulins with the two protein kinases are shown in Figure 6. Metabolically labeled calmodulins were produced by growing *E. coli* harboring plasmids containing the various calmodulin genes in the presence of [^{35}S]sulfate. Each of the labeled calmodulins was purified to chemical homogeneity. Under the conditions used, the purified calmodulins have similar specific activities (approximately 150 cpm/fmol). Equal amounts of protein kinase II (0.75 μg) and myosin light chain kinase (1.00 μg) were loaded on four different gels which were taken through the electrophoresis, staining, renaturation, and incubation procedures at the same time and under identical conditions. The same amount of radioactivity (calmodulin) was added to each gel incubation mixture (4×10^6 cpm, or 200–300 pmol of a given SYNCAM on each gel). Each panel shown is the autoradiogram after 24 hour exposure for each gel at −80°C.

properties of SYNCAM-8 calmodulin are similar to those of the wild-type SYNCAM calmodulin in circular dichroism spectroscopy[13]; (5) the macroscopic calcium-binding properties of SYNCAM-8 calmodulin are unchanged[64] and initial NMR spectroscopy studies indicate that the interactions between the central helix and the C-terminal domain are conserved[65] (J. Krebs and D.M. Watterson, unpublished results).

Despite the apparent maintenance of overall structural similarities between SYNCAM and SYNCAM-8 calmodulins, clearly they are functionally different. The functional effect of the central helix charge reversal mutation is to alter MLCK and NAD kinase activation,[13] but there is no detectable effect, under the conditions used, on phosphodiesterase[13] or calmodulin-dependent protein kinase II activation (Fig. 6). If there were a major effect on the overall structure of calmodulin, all of the calmodulin activities would be affected, as is found for some chemical modifications of calmodulin.[23,26] Thus, within the context of what is known about the effects of various treatments on native calmodulin structure and function,[3,4] the selective functional effects of the helix charge reversal mutation in SYNCAM-8 calmodulin are consistent with a preservation of significant native structure. Further, other selected mutations in the central helix have little or no effect on the ability of calmodulin to activate myosin light chain kinase,[15,16,64] thus demonstrating that simply any change in the central helix does not result in an altered functional interaction between calmodulin and this enzyme. As discussed below, the effect of the central helix charge reversal found in SYNCAM-8 on the activation of myosin light chain kinase is reflected in a diminished binding activity in a gel overlay assay (Fig. 7).

Overall, the results suggest that the altered interaction of SYNCAM-8 with myosin light chain kinase might involve localized changes in the charge properties of calmodulin. Indeed, calculations of the electrostatic potential surface for SYNCAM-8 calmodulin shows substantial reduction in negative electrostatic potential near the central part of the molecule relative to native SYNCAM calmodulin (Fig. 5B).

Investigation of the Possible Functional Importance of Charge Features, Carboxy-Terminal Domain Helix Residues 118–120

The four primary structure motifs of calmodulin internally repeat in the amino acid sequence[42] such that the acidic cluster at residues 82–84 aligns with another acidic cluster at residues 118–120 (Fig. 2). In the structure[37] (Fig. 1), both clusters are located within α-helices. Similar to the surface near residues 82–84, one of the largest continuous regions of

TABLE II. Amino Acid Compositions of SYNCAM-12A and SYNCAM-18A Calmodulins

Amino acid	SYNCAM calmodulin*	SYNCAM-8 calmodulin†*	SYNCAM-12A calmodulin†	SYNCAM-18A calmodulin†
Aspartic acid	25	25	26.6 (24)	26.8 (24)
Glutamic acid	26	23	24.4 (24)	21.6 (21)
Serine	4	4	3.5 (4)	3.5 (4)
Glycine	11	11	10.9 (11)	10.7 (11)
Histidine	1	1	1.0 (1)	1.0 (1)
Arginine	5	5	5.2 (5)	5.1 (5)
Threonine	10	10	9.1 (10)	9.0 (10)
Alanine	11	11	10.8 (11)	10.8 (11)
Proline	2	2	2.8 (2)	2.7 (2)
Tyrosine	1	1	1.0 (1)	1.0 (1)
Valine	9	9	8.5 (9)	8.6 (9)
Methionine	8	8	7.3 (8)	7.4 (8)
Isoleucine	6	6	5.8 (6)	5.8 (6)
Leucine	11	11	10.9 (11)	10.9 (11)
Phenylalanine	9	9	8.6 (9)	8.6 (9)
Lysine	9	12	11.7 (12)	14.6 (15)
Cysteine	0	0	ND‡ (0)	ND (0)
Tryptophan	0	0	ND (0)	ND (0)

*Residues/molecule calculated from the amino acid sequences.[13,38]
†Moles amino acid/mole of protein assuming a molecular weight of 16,700 are shown. The numbers in parentheses indicate the expected values based on the amino acid sequence (Fig. 2).
‡ND, not determined.

high negative electrostatic potential is located near residues 118–120 (Fig. 4). Given these similarities in charge and structure, the effects of reversing the charge at residues 118–120 were investigated.

The calculated electrostatic stabilization for changing the Asp-Glu-Glu sequence at residues 118–120 to lysines (i.e., production of SYNCAM-12A calmodulin) is less than for SYNCAM-8 calmodulin despite the same change in net charge (Table I). The differences in calculated electrostatic stabilization may reflect the relative molecular locations of the charge clusters. In SYNCAM-8 calmodulin, the charge reversal occurs near the center of the negative region that extends over much of the molecular surface, whereas the altered charges in SYNCAM-12A are located at the edge of the charged surface (Fig. 4).

In contrast to the comparatively small change in the calculated electrostatic stabilization, the charge reversal in SYNCAM-12A results in a neutralization of nearly half of the negative electrostatic potential surface on the carboxy-terminal domain (Fig. 5C). Given the large predicted changes in electrostatic properties, a calmodulin containing this charge cluster reversal was designed and produced by cassette-based site-specific mutagenesis. The amino acid composition of the purified protein is given in Table II and the DNA sequence elucidated for the gene is indicated in Figure 2B.

As seen in Figure 6A, SYNCAM-12A calmodulin has a diminished ability to activate myosin light chain kinase, but the alteration in activity is distinct from that produced by the acidic cluster mutation found in SYNCAM-8 calmodulin. While both show similar reduction in the maximal activity obtained for myosin light chain kinase, the calmodulin concentration required for half maximal activation is less for SYNCAM-12A calmodulin than for SYNCAM-8 calmodulin. These results suggest that relative to SYNCAM calmodulin, greater alterations of calmodulin–myosin light chain kinase interactions exist for SYNCAM-8 than for SYNCAM-12A calmodulin. This possibility was addressed by using biosynthetically labeled SYNCAM, SYNCAM-8, and SYNCAM-12A calmodulins in a ligand-binding assay developed as a screening procedure for the detection of calmodulin-binding proteins in cell extracts and recombinant DNA clones.[50,51] As shown in Figure 7, the results are consistent with diminished binding by SYNCAM-8 calmodulin to myosin light chain kinase compared to the binding of SYNCAM-12A and SYNCAM calmodulins.

Because calmodulin-dependent protein kinase II has a calmodulin-binding domain with an amino acid sequence and potential secondary structure similar to myosin light chain kinase[66] but responds differentially to calmodulin mutations,[15] the interaction of SYNCAM, SYNCAM-8, and SYNCAM-12A calmodulins with calmodulin-dependent protein kinase II was also examined. Calmodulin-dependent activation of both calmodulin-dependent kinase II and myosin light chain kinase involves formation of a calmodulin–enzyme complex (for review see ref. 66). Although the regions of these enzymes that bind calmodulin have similar structural features, the mechanisms of activation by calmodulin are different.[66,67] Activation of protein kinase II appears to result in a stimulation of enzyme autophosphorylation with a concomitant loss of sensitivity to calmodulin,[49,68–70] while myosin light chain kinase

does not appear to undergo significant autophosphorylation.[31] In contrast to the effect on MLCK activation, mutation of the central helix cluster at residues 82–84 (SYNCAM-8 calmodulin) only slightly affects activation of calmodulin-dependent protein kinase II (Fig. 6B). However, the charge reversal at residues 118–120 (SYNCAM-12A) has a significant effect on activation of calmodulin-dependent protein kinase II (Fig. 6).

In a binding assay similar to that described above for myosin light chain kinase, SYNCAM-8 and SYNCAM-12A demonstrate different interactions with calmodulin-dependent protein kinase II (Fig. 7). The native rat brain calmodulin-dependent protein kinase II used in these studies is a dodecamer composed of at least two types of subunits in a ratio of approximately 3 to 1.[66] Therefore, the studies described here cannot be used to draw conclusions concerning any apparent difference or similarity in calmodulin-binding activity between the two bands seen on the gel in Figure 7, although comparisons can be made among calmodulins with the same gel band.

In summary, two calmodulin-regulated protein kinases that differ in their mechanism of calmodulin regulation respond differently to the charge reversal mutations in calmodulin. In addition, the protein-binding data combined with the enzyme activator data, especially with calmodulin-dependent protein kinase II, indicate that the effects of the cluster charge reversal mutations are more complex than what might be inferred from either data alone.

Combination of Cluster Charge Reversals

The calmodulin-dependent activity and binding assays raised the question of whether the combination of the cluster mutations would be additive in their functional effects or suggest localized effects of the individual cluster mutations. In order to address this question, another mutant calmodulin, SYNCAM-18A, which incorporates the changes found in SYNCAM-8 and SYNCAM-12A, was constructed and produced as described in Materials and Methods. Changing six acidic residues (residues 82–84 and 118–120) to lysine greatly reduces the overall molecular charge to −12.0, and slightly stabilizes the molecule (Table I). The electrostatic potential surface of SYNCAM-18A calmodulin additively shows the localized changes seen in SYNCAM-8 and SYNCAM-12A calmodulins relative to SYNCAM calmodulin (Fig. 5D). Although less information is available concerning the overall structure of SYNCAM-18A calmodulin, the protein was purified by exactly the same protocol that was used for the purification of SYNCAM, SYNCAM-8, and SYNCAM-12A calmodulins. This protocol is based on the general physical properties of calmodulin and utilizes a calcium-dependent adsorption chromatography step. The fact that SYNCAM-18A could be purified by this protocol and retains the ability to activate phosphodiesterase (data not shown) in a calcium-dependent manner is consistent with a retention of a significant amount of the overall structural features of native calmodulin.

The functional effects of combining the mutations of SYNCAM-8 and SYNCAM-12A calmodulins into a single protein, SYNCAM-18A calmodulin, are shown in Figures 6 and 7. Activation of myosin light chain kinase by SYNCAM-18A appears to combine the activity losses seen with SYNCAM-8 and SYNCAM-12A (Fig. 6A). However, SYNCAM-18A activation of calmodulin-dependent protein kinase II is similar to that found with the single charge cluster mutation of SYNCAM-12A (Fig. 6B). Therefore, activation of MLCK appears to be sensitive to charge reversal in both the central helix and carboxy-terminal domain, while activation of calmodulin-dependent protein kinase II is more sensitive to charge alteration in the carboxy-terminal domain. The gel overlay binding assay shows similar decreases in binding in MLCK for SYNCAM-18A and SYNCAM-8, indicating that the charge clusters, especially that in the central helix, are important for calmodulin–MLCK binding. As shown by the dramatic loss in binding of SYNCAM-18A to calmodulin-dependent protein kinase II, diminution of calmodulin binding to this enzyme increases with the number of charge reversals, a fact not as clearly seen if only enzyme activation were examined.

In summary, the computational, enzyme activation, and gel overlay binding results on each of the cluster charge reversal mutations clearly demonstrate that alteration of charge features on calmodulin differentially affects binding to and activation of myosin light chain kinase and calmodulin-dependent protein kinase II.

DISCUSSION

The studies described here, in which mutations that could disrupt the localized charge properties of calmodulin were identified by computational analysis and tested empirically by combining mutagenesis and activity analysis, demonstrate that the charge properties of calmodulin are one of several properties of calmodulin that are functionally important. In the case of myosin light chain kinase, calmodulins with lysines at residues 82–84 or 118–120 are diminished in their ability to activate the enzyme. The presence of lysines at residues 82–84 results in a localized disruption of the negative potential surface of calmodulin and alters its ability to activate and interact with myosin light chain kinase, without disrupting the overall structure or macroscopic calcium-binding constants of calmodulin.[13,15,60,64] However, a clear alteration in the ability of calmodulin to activate the calmodulin-dependent protein kinase II is manifest only with the presence of lysines in the residue 118–120 region. In

contrast to the differential effect on protein kinases, the presence of lysines at residues 82–84 or 118–120 does not have a major effect on phosphodiesterase activity under the conditions used. Altogether, the results presented here and elsewhere[13,15,17] demonstrate that a number of perturbations of residues in calmodulin helices can be accommodated by calmodulin and the enzymes it regulates, and are in agreement with conclusions, based on analysis of crystal structures of mutant lysozymes,[62] about solvent-exposed residues within α-helices of proteins. The results summarized here are also consistent with a model in which the asymmetric distribution of uncompensated negative charge on calmodulin may be important in restricting the number of possible conformational states of cal- modulin,[8,9] and raise the possibility of key acidic residues in the 82–84 or 118–120 region of calmodulin being functionally important in the activation of myosin light chain kinase, a calmodulin-regulated enzyme with a preponderance of basic and hydrophobic residues in its amphiphilic calmodulin binding site.[31]

Several computational approaches and methods have been used to explore the role of electrostatics in proteins or protein–protein interactions.[56,72,73] In most cases, application of the method was facilitated by the availability of molecular models based on systems where crystallographic data had been obtained for each component of a complex, or the complex itself. In the present study, the possible role of electrostatics in an effector protein (calmodulin)–enzyme system was investigated with only a model of the effector protein. Therefore, the approach is an exploratory one where, through the use of site-specific mutagenesis and functional analysis, the functional contribution of certain charge features of one protein of the complex was examined. While the results provide empirical functional data that demonstrate the feasibility of the approach and provide a focus for subsequent mutagenesis and functional analyses, a full description of the system requires electrostatic calculations on calmodulin–enzyme complexes or, as a first step, calmodulin–peptide complexes. The availability of a crystallographic structure for a calmodulin–peptide or calmodulin–enzyme complex would facilitate such calculations. Clearly, regardless of the inherent limitations of the molecular modeling aspects of this study, the use of the modeling to generate testable hypotheses and the empirical testing of their feasibility have allowed the development of a reasonable model of how certain features of calmodulin structure are related to its ability to function as a component of a eukaryotic cell signal transduction complex.

When the results summarized here are evaluated in the context of previous studies of calmodulin structure and function (for a summary, see refs. 2–4,8,9,37) as well as analyses of electrostatics in protein–protein interaction (for examples, see refs. 57–59, 74–77), there are several testable hypotheses that can be raised concerning the possible roles that negative electrostatic potential surfaces might play in calmodulin function. One hypothesis is that the assymetric distribution of the electrostatic surface potential, with the localized areas of relatively high density of uncompensated negative charge, might be important in conformational restriction or selection. The electrostatic repulsion between similarly charged residues could function to keep like-charged regions of the molecule away from each other, thereby restricting the number of possible conformations the molecule can assume in solution. The predominate localization of the negative electrostatic potential on a particular face of the molecule could promote motions of the molecule that might expand the negatively charged face to relieve electrostatic repulsion. In other words, conformations that require bringing the similarly charged regions closer together would not be favored. As can be inferred from an inspection of Figure 3, this restriction could be in a plane parallel or perpendicular to the long axis of the central helix.

Initial time-resolved fluorescence studies of isofunctional structural mutants of calmodulin[8,9,78] are consistent with a mechanism in which calmodulin exists in solution as a set of rapidly interchanging conformational states, with calcium selecting some of these conformations and decreasing the exchange rate between the multiple conformations. More recent time-resolved fluorescence studies (M. Chabbert, D.M. Watterson, and F.G. Prendergast, in preparation), in which the effects of calmodulin interaction with a peptide analog of the calmodulin binding site of MLCK[31] were examined, suggest that binding of the calcium–calmodulin complex to the peptide may result in a further selection of conformational states. A corollary of this model would be that molecular flexibility might be altered with mutations that alter the features of this asymmetric charge property. Calmodulins with altered charge features (this report) and isofunctional structural mutants with tryptophans introduced as internal reporter groups[8,9,78] could be combined in future studies in order to address experimentally this possibility. Altogether, the results described here and those employing time-resolved fluorescence spectroscopy are consistent with one function of an asymmetric negative charge distribution being to restrict the initial population of conformations in which calmodulin can exist. Such a situation also could add an element of discrimination to the calmodulin structure, thereby allowing the various calmodulin-regulated enzymes to recognize unique as well as common structural features on calmodulin.

In terms of potential physiological significance, the conformational restriction role for an asymmetric negative electrostatic potential surface might be

in affecting the kinetics of correct subunit assembly *in vivo*. This type of electrostatic effect might be important in promoting correct pairing of calmodulin with calmodulin-regulated enzymes during development and cellular turnover. Although calmodulin can form functional complexes *in vitro* with proteins that are physiological targets for other calcium-binding proteins,[79] there is correct pairing in the cell. In addition, calmodulin is one of the many genes in a eukaryotic cell that are essential for survival.[80,81] Therefore, there is a strict *in vivo* time frame for the assembly of properly assembled complexes. As noted by Levinthal,[82] there must be restrictions on the number of allowed conformations that a given protein can assume if an individual polypeptide chain is to fold into its native conformation in a reasonable amount of time, i.e., sampling of all allowed conformational states is not feasible. This problem is accentuated with calmodulin, an essential nonenzymatic effector protein that must coordinate its biosynthesis with other proteins if functional complexes are to be assembled within the time frame of cell division or adaptation. This possible conformational restriction function of calmodulin electrostatic properties would probably not be affected by single charge mutations within the regions examined in this study, although the triple charge mutations described here could possibly affect this function. In this regard, clustering of excess negative charge might result in a protection against point mutations having a deleterious effect on all calmodulin-regulated processes.

Another possible role for calmodulin electrostatics in the interaction with protein kinases might be for catalytic function or stabilization of the more intimate, productive protein:protein complex. If such point electrostatic interactions are important, then altering a key single residue, or set of single residues, would be expected to alter activity. Subsequent, more detailed mutagenesis studies of calmodulin based on the model presented here have resulted in the identification of glutamic acid 84 and glutamic acid 120 in these charge clusters as being functionally important acidic residues (Watterson, Lukas, Kwiatkowski and Craig, in preparation). Thus, the electrostatic properties of calmodulin that are functionally important might include the overall electrostatic potential surface, which would be perturbed by cluster mutations, and key charged residues whose functional importance can be detected by point, or sets of point, mutations.

CONCLUSIONS

The amino- and carboxy-terminal domains and the four calcium-binding domains of calmodulin are not functionally equivalent, despite their high degree of sequence and structural similarity. For example, the binding of calcium to the four calcium-selective sites[8] appears to be cooperative and cannot be adequately described by models that assume four identical sites or two pairs of identical sites.[8,64] Similarly, the structural features of calmodulin that are important for enzyme activation vary among calmodulin-regulated enzymes (for examples based on mutagenesis see references 12,13,15,17,19,83,88, and this report). The electrostatic potential surface of calmodulin described in this report is a feature that might contribute asymmetric properties to the protein. Clearly, the ability to produce calmodulins with altered function based on the calculations described here demonstrates the functional importance of maintaining calmodulin's charge properties. These results also indicate that one basis of the functional effects found as a result of the central helix perturbations reported by Craig et al.[13] may be the alteration of the localized charge properties of the central helix, indicate that features of the calmodulin central helix in addition to secondary structure[17] and length[19] must be considered in models of its functional roles, and suggest that similar, although not identical, features of calmodulin may be important in the interaction between calmodulin and the type II calmodulin-dependent protein kinase.

ACKNOWLEDGMENTS

This research was supported in part by N.I.H. Grant GM30861 (DMW) and NSF Grant DMB-8502706 (MMK). We thank Paul Matrisian, Augustine Smith, B.C. Finzel, and S.J. Kimatian for their invaluable assistance with these studies and Janis Elsner and Cindy Reeder for their help with the preparation of this manuscript.

REFERENCES

1. Van Eldik, L.J., Zendegui, J.G., Marshak, D.R., and Watterson, D.M. Calcium-binding proteins and the molecular basis of calcium action. Int. Rev. Cytol. 77:1–61, 1982.
2. Roberts, D.M., Lukas, T.J., and Watterson, D.M. Structure, function, and mechanism of action of calmodulin. CRC Crit. Rev. Plant Sci. 4:311–339, 1986.
3. Klee, C.B., and Vanaman, T.C. Calmodulin. Adv. Protein Chem. 35:213–321, 1982.
4. Cohen, P., and Klee, C.B. "Calmodulin." Amsterdam: Elsevier, 1988.
5. Huang, B., Mengersen, A., and Lee, V.D. Molecular cloning of cDNA for caltractin, a basal body-associated Ca^{2+}-binding protein: Homology in its protein sequence with calmodulin and the yeast CDC31 gene product. J. Cell Biol. 107:133–140, 1988.
6. Collins, J.H., Greaser, M.L., Potter, J.D., and Horn, M.J. Determination of the amino acid sequence of troponin C from rabbit skeletal muscle. J. Biol. Chem. 252:6356–6362, 1977.
7. Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K., and Hartshorne, D.J. Modulator protein as a component of the myosin light chain kinase from chicken gizzard. Biochemistry 17:253–258, 1978.
8. Kilhoffer, M.-C., Roberts, D.M., Adibi, A.O., Watterson, D.M., and Haiech, J. Investigation of the mechanism of calcium binding to calmodulin. Use of an isofunctional mutant with a tryptophan introduced by site-directed mutagenesis. J. Biol. Chem. 263:17023–17029, 1988.
9. Chabbert, M., Kilhoffer, M.C., Watterson, D.M., Haiech, J., and Lami, H. Time-resolved fluorescence study of VU-9

calmodulin, an engineered calmodulin possessing a single tryptophan residue. Biochemistry, 28:6093–6098, 1989.
10. Olwin, B.B., and Storm, D.R. Calcium binding to complexes of calmodulin and calmodulin binding proteins. Biochemistry 24:8081–8086, 1985.
11. Huang, C.Y., Chau, V., Chock, P.B., Wang, J.H., and Sharma, R.K. Mechanism of activation of cyclic nucleotide phosphodiesterase: Requirement of the binding of four Ca^{2+} to calmodulin for activation. Proc. Natl. Acad. Sci. U.S.A. 78:871–874, 1981.
12. Roberts, D.M., Rowe, P.M., Siegel, F.L., Lukas, T.J., and Watterson, D.M. Trimethyllysine and protein function. Effect of methylation and mutagenesis of lysine 115 of calmodulin on NAD kinase activation. J. Biol. Chem. 261: 1491–1494, 1986.
13. Craig, T.A., Watterson, D.M., Prendergast, F.G., Haiech, J., and Roberts, D.M. Site-specific mutagenesis of the α-helices of calmodulin. Effects of altering a charge cluster in the helix that links the two halves of calmodulin. J. Biol. Chem. 262:3278–3284, 1987.
14. Craig, T.A., Watterson, D.M., and Hinrichsen, R.D. Analysis of a mutant *Paramecium* with a nonlethal selective alteration in calmodulin regulation and a defective calcium-dependent potassium conductance. J. Cell Biol. 105: 143a, 1987.
15. Craig, T.A., Roberts, D.M., King, M.M., Lukas, T.J., Watterson, D.M., and Prendergast, F.G. Site-specific mutagenesis of central α-helix of calmodulin: Effects of altering charge clusters, substitution of prolines and insertion of troponin C-like segment. Fed. Proc. 46:2001, 1987.
16. Lukas, T.J., Haiech, J., Lau, W., Craig, T.A., Zimmer, W.E., Shattuck, R.L., Shoemaker, M.O., and Watterson, D.M. Calmodulin and calmodulin-regulated protein kinases as transducers of intracellular calcium signals. Cold Spring Harbor Symp. Quant. Biol. 53:185–193, 1988.
17. Putkey, J.A., Ono, T., VanBerkum, M.F.A., and Means, A.R. Functional significance of the central helix in calmodulin. J. Biol. Chem. 263:11242–11249, 1988.
18. Wilson, E., Craig, T.A., and Watterson, D.M. Similarities between the ion channel associated calmodulin binding protein and the beta subunit of GTP-binding proteins. J. Cell Biol. 107:142a, 1988.
19. Persechini, A., Hardy, D.O., Blumenthal, D.K., Jarrett, H.W., and Kretsinger, R.H. The effects on enzyme activation of genetically-engineered amino acid deletions in the calmodulin long helix. Biophys. J. 53:252a, 1988.
20. Kuznicki, J., Graberek, Z., Brzeska, H., Drabikowski, W., and Cohen, P. Stimulation of enzyme activities by fragments of calmodulin. FEBS Lett. 130:141–145, 1981.
21. Newton, D.L., Oldewurtel, M.D., Krinks, M.H., Shiloach, J., and Klee, C.B. Agonist and antagonist properties of calmodulin fragments. J. Biol. Chem. 259:4419–4426, 1984.
22. Newton, D.L., and Klee, C.B. CAPP-calmodulin: A potent competitive inhibitor of calmodulin actions. FEBS Lett. 165:269–272, 1984.
23. Jackson, A.E., and Puett, D. Specific acylation of calmodulin. J. Biol. Chem. 259:14985–14992, 1984.
24. Mann, D.M., and Vanaman, T.C. Topographical mapping of calmodulin-target enzyme interaction domains. J. Biol. Chem. 264:2373–2378, 1989.
25. Andreasen, T.J., Keller, C.H., LaPorte, D.C., Edelman, A.M., and Storm, D.R. Preparation of azidocalmodulin: A photoaffinity label for calmodulin-binding proteins. Proc. Natl. Acad. Sci. U.S.A. 78:2782–2785, 1981.
26. Lukas, T.J., Marshak, D.R., and Watterson, D.M. Drug-protein interactions: Isolation and characterization of covalent adducts of phenoxybenzamine and calmodulin. Biochemistry 24:151–157, 1985.
27. Guerini, D., Krebs, J., and Carafoli, E. Stimulation of erythrocyte Ca^{2+} ATPase and bovine cyclic nucleotide phosphodiesterase by chemically modified calmodulin. Eur. J. Biochem. 170:35, 1987.
28. McDowell, L., Sanyal, G., and Prendergast, F.G. Probable role of amphiphilicity in the binding of mastoparan to calmodulin. Biochemistry 24:2979–2984, 1985.
29. Milos, M., Schaer, J.-J., Comte, M., and Cox, J.A. Microcalorimetric investigation of the interactions in the ternary complex calmodulin-calcium-melittin. J. Biol. Chem. 262:2746–2749, 1987.
30. O'Neil, K.T., Wolfe, H.R., Jr., Erickson-Viitanen, S., and DeGrado, W.F. Fluorescence properties of calmodulin-binding peptides reflect alpha-helical periodicity. Science 236:1454–1456, 1987.
31. Lukas, T.J., Burgess, W.H., Prendergast, F.G., Lau, W., and Watterson, D.M. Calmodulin binding domains: Characterization of a phosphorylation and calmodulin binding site from myosin light chain kinase. Biochemistry 25:1458–1464, 1986.
32. Malencik, D.A., and Anderson, S.R. Calmodulin-linked equilibria in smooth muscle myosin light chain kinase. Biochemistry 25:709–721, 1986.
33. Klevit, R.E., and Blumenthal, D.K. 1H NMR studies of calmodulin-peptide interactions. In: "Calcium-Binding Proteins in Health and Disease." A.W. Norman, T.C. Vanaman, and A.R. Means, eds. San Diego: Academic Press, 1987:333–347.
34. Blumenthal, D.K., and Stull, J.T. Effects of pH, ionic strength, and temperature on activation by calmodulin and catalytic activity of myosin light chain kinase. Biochemistry 21:2386–2391, 1982.
35. Hubbard, M.J., and Klee, C.B. Calmodulin binding by calcineurin. Ligand-induced renaturation of protein immobilized on nitrocellulose. J. Biol. Chem. 262:15062–15070, 1987.
36. Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R., and Cook, W.J. Three-dimensional structure of calmodulin. Nature (London) 315:37–40, 1985.
37. Babu, Y.S., Bugg, C.E., and Cook, W.J. Structure of calmodulin refined at 2.2 Å resolution. J. Mol. Biol. 204:191–204, 1988.
38. Roberts, D.M., Crea, R., Malecha, M., Alvarado-Urbina, G., Chiarello, R.H., and Watterson, D.M. Chemical synthesis and expression of a calmodulin gene designed for site-specific mutagenesis. Biochemistry 24:5090–5098, 1985.
39. Roberts, D.M., Burgess, W.H., and Watterson, D.M. Comparison of the NAD kinase and myosin light chain kinase activator properties of vertebrate, higher plant, and algal calmodulins. Plant Physiol. 75:796–798, 1984.
40. Schleicher, M., Lukas, T.J., and Watterson, D.M. Further characterization of calmodulin from the monocotyledon barley (Hordeum vulgare). Plant Physiol. 73:666–670, 1983.
41. Marshak, D.R., Clarke, M., Roberts, D.M., and Watterson, D.M. Structural and functional properties of calmodulin from the eukaryotic microorganism *Dictyostelium discoideum*. Biochemistry 23:2891–2899, 1984.
42. Watterson, D.M., Sharief, F., and Vanaman, T.C. The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. J. Biol. Chem. 255:962–975, 1980.
43. Watterson, D.M., Burgess, W.H., Lukas, T.J., Iverson, D., Marshak, D.R., Schleicher, M., Erickson, B.W., Fok, K.-F., and Van Eldik, L.J. Towards a molecular and atomic anatomy of calmodulin and calmodulin-binding proteins. In: "Advances in Cyclic Nucleotide and Protein Phosphorylation Research." S.J. Strada, and W.J. Thompson, eds. Raven Press, New York: Raven, 1984:205–226.
44. Lukas, T.J., Iverson, D.B., Schleicher, M., and Watterson, D.M. Structural characterization of a higher plant calmodulin *Spinacia oleracea*. Plant Physiol. 75:788–795, 1984.
45. Roberts, D.M., Zimmer, W.E., and Watterson, D.M. The use of synthetic oligodeoxyribonucleotides in the examination of calmodulin gene and protein structure and function. Meth. Enzymol. 139:290–303, 1987.
46. Zimmer, W.E., Schloss, J.A., Silflow, C.D., Youngblom, J., and Watterson, D.M. Structural organization, DNA sequence and expression of the calmodulin gene. J. Biol. Chem. 263:19370–19383, 1988.
47. Schaefer, W.H., Lukas, T.J., Blair, I.A., Schultz, J.E., and Watterson, D.M. Amino acid sequence of a novel calmodulin from *Paramecium tetraurelia* that contains dimethyllysine in the first domain. J. Biol. Chem. 262:1025–1029, 1987.
48. Bidlingmeyer, B.A., Cohen, S.A., and Tarvin, T.L. Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. 336:93–104, 1984.
49. Kwiatkowski, A.P., Shell, D.J., and King, M.M. The role of autophosphorylation in activation of the type II calmodu-

lin-dependent protein kinase. J. Biol. Chem. 263:6484–6486, 1988.
50. Asselin, J., Phaneuf, S., Watterson, D.M., and Haiech, J. Metabolically 35S-labeled recombinant calmodulin as a ligand for the detection of calmodulin-binding proteins. Anal. Biochem. 178:141–147, 1989.
51. Burgess, W.H., Watterson, D.M., and Van Eldik, L.J. Identification of calmodulin-binding proteins in chicken embryo fibroblasts. J. Cell. Biol. 99:550–557, 1984.
52. Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., Jr., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., and Tasumi, M. The Protein Data Bank: A computer-based archival file for macromolecular structures. J. Mol. Biol. 112:535–542, 1977.
53. Jones, T.A., and Thirup, S. Using known substructures in protein model building and crystallography. EMBO J. 5: 819–822, 1986.
54. Ponder, J.W., and Richards, F.M. Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. J. Mol. Biol. 193:775–791, 1987.
55. Weiner, S.J., Kollman, P.A., Nguyen, D.T., and Case, D.A. An all atom force field for simulations of proteins and nucleic acids. J. Comp. Chem. 7:230–252, 1986.
56. Matthew, J.B., and Gurd, F.R.N. Calculation of electrostatic interactions in proteins. Meth. Enzymol. 130:413–436, 1986.
57. Weber, P.C., and Tollin, G. Electrostatic interactions during electron transfer reactions between c-type cytochromes and flavodoxin. J. Biol. Chem. 260:5568–5573, 1985.
58. Wendoloski, J.J., and Matthew, J.B. Molecular dynamics effects on protein electrostatics. Proteins: Structure, Function, Genet., 5:313–321, 1989.
59. Warwicker, J. Investigating protein-protein interaction surfaces using a reduced stereochemical and electrostatic model. J. Mol. Biol. 206:381–395, 1989.
60. Lukas, T.J., Craig, T.A., Roberts, D.M., Watterson, D.M., Haiech, J., and Prendergast, F.G. An interdisciplinary approach to the molecular mechanisms of calmodulin action: Comparative biochemistry, site-specific mutagenesis, and protein engineering. In: "Calcium-Binding Proteins in Health and Disease." A.W. Norman, T.C. Vanaman, and A.R. Means, eds. New York: Academic Press, 533–543, 1987.
61. Maxfield, F.R., and Scheraga, H.A. The effect of neighboring charges on the helix forming ability of charged amino acids in proteins. Macromolecules 8:491–493, 1975.
62. Alber, T., Bell, J.A., Dao-Pin, S., Nicholson, H., Wozniak, J.A., Cook, S., and Matthews, B.W. Replacements of Pro86 in Phage T4 lysozyme extend an alpha-helix but do not alter protein stability. Science 239:631–635, 1988.
63. Grutter, M.G., and Matthews, B.W. Amino acid substitutions far from the active site of bacteriophage T4 lysozyme reduce catalytic activity and suggest that the C-terminal lobe of the enzyme participates in substrate binding. J. Mol. Biol. 154:525–535, 1982.
64. Haiech, J., Kilhoffer, M.-C., Craig, T.A., Lukas, T.J., Wilson, E., Guerra-Santos, L., and Watterson, D.M. Mutant analysis approaches to understanding calcium signal transduction through calmodulin and calmodulin regulated enzymes. In: "Proceedings of First European Symposium on Calcium Binding Proteins." D.E.M. Lawson, and R. Pochet, eds. New York: Plenum Press, in press.
65. Krebs, J., Vorherr, T., James, P., Carafoli, E., Craig, T.A., and Watterson, D.M. Structural details of the interaction of calmodulin with the plasma membrane Ca^{2+}-ATPase. In: "Proceedings of First European Symposium on Calcium Binding Proteins." D.E.M. Lawson and R. Pochet, eds. New York: Plenum Press, in press.
66. Schulman, H., and Lou, L.L. Multifunctional Ca^{2+}/calmodulin-dependent protein kinase: Domain structure and regulation. TIBS 14:62–66, 1989.
67. Miller, S.G., and Kennedy, M.B. Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: A Ca^{2+}-triggered molecular switch. Cell 44:861–870, 1986.
68. Lai, Y., Nairn, A.C., and Greengard, P. Autophosphorylation reversibly regulates the Ca^{2+}/calmodulin-dependence of Ca^{2+}/calmodulin-dependent protein kinase II. Proc. Natl. Acad. Sci. U.S.A. 83:4253–4257, 1986.
69. Schworer, C.M., Colbran, R.J., and Soderling, T.R. Reversible generation of a Ca^{2+}-independent form of Ca^{2+} (calmodulin)-dependent protein kinase II by an autophosphorylation mechanism. J. Biol. Chem. 261:8581–8584, 1986.
70. Lou, L.L., Lloyd, S.J., and Schulman, H. Activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme. Proc. Natl. Acad. Sci. U.S.A. 83:9497–9501, 1986.
71. Matsumura, M., Becktel, W.J., and Matthews, B.W. Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. Nature (London) 334: 406–410, 1988.
72. Warwicker, J., and Watson, H.C. Calculation of the electric potential in the active site cleft due to alpha-helix dipoles. J. Mol. Biol. 157:671–679, 1982.
73. Zauhar, R.J., and Morgan, R.S. A new method for computing the macromolecular electric potential. J. Mol. Biol. 186: 815–820, 1985.
74. Matthew, J.B., Weber, P.C., Salemme, F.R., and Richards, F.M. Electrostatic orientation during electron transfer between flavodoxin and cytochrome c. Nature (London) 301: 169–171, 1983.
75. Matthew, J.B., and Ohlendorf, D.H. Electrostatic deformation of DNA by a DNA-binding protein. J. Biol. Chem. 260:5860–5862, 1985.
76. Salemme, F.R. An hypothetical structure for an intermolecular election complex of cytochromes c and b5. J. Mol. Biol. 102:563–568, 1976.
77. Warwicker, J., Ollis, D., Richards, F.M., and Steitz, T.A. Electrostatic field of the large fragment of *Escherichia coli* DNA polymerase I. J. Mol. Biol. 186:645–649, 1985.
78. Kilhoffer, M.-C., Roberts, D.M., Adibi, A., Watterson, D.M., and Haiech, J. Fluorescence characterization of VU-9 calmodulin an engineered calmodulin with one tryptophan in calcium binding domain III. Biochemistry 28: 6086–6092, 1989.
79. Amphlett, G.W., Vanaman, T.C., and Perry, S.V. Effect of the troponin C-like protein from bovine brain (brain modulator protein) on the Mg^{2+}-stimulated ATPase of skeletal muscle actomyosin. FEBS Lett. 72:163–168, 1976.
80. Davis, T.N., Urdea, M.S., Masiarz, F.R., and Thorner, J. Isolation of the yeast calmodulin gene: Calmodulin is an essential protein. Cell 47:423–431, 1986.
81. Takeda, T., and Yamamoto, M. Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. U.S.A. 84:3580–3584, 1987.
82. Levinthal, C. Are there pathways for protein folding? J. Chim. Phys. 65:44–45, 1968.
83. Haiech, J., Predeleanu, R., Watterson, D.M., Ladant, D., Bellalou, J., Ullmann, A., and Barzu, O. Affinity-based chromatography utilizing genetically engineered proteins. Interaction of *Bordetella pertussis* adenylate cyclase with calmodulin. J. Biol. Chem. 263:4259–4262, 1988.
84. Lukas, T.J., Wiggins, M.E., and Watterson, D.M. Amino acid sequence of a novel calmodulin from the unicellular alga *Chlamydomonas*. Plant Physiol. 78:477–483, 1985.
85. Tschudi, C., Young, A.S., Ruben, L., Patton, C.L., and Richards, F.F. Calmodulin genes in trypanosomes are tandemly repeated and produce multiple mRNAs with a common 5′ leader sequence. Proc. Natl. Acad. Sci. U.S.A. 82: 3998–4002, 1985.
86. Toda, H., Yazawa, M., Kondo, K., Honma, T., Narita, K., and Yagi, K. Amino acid sequence of calmodulin from scallop (*Patinopecten*) adductor muscle. J. Biochem. 90:1493–1505, 1981.
87. Smith, V.L., Doyle, K.E., Maune, J.F., Munjaal, R.P., and Beckingham, K. Structure and sequence of the *Drosophila melanogaster* calmodulin gene. J. Mol. Biol. 196:471–485, 1987.
88. Schaefer, W.H., Hinrichsen, R.D., Burgess-Cassler, A., Kung, C., Blair, I.A., and Watterson, D.M. A mutant *Paramecium* with a defective calcium-dependent potassium conductance has an altered calmodulin: A nonlethal selective alteration in calmodulin regulation. Proc. Natl. Acad. Sci. U.S.A. 84:3931–3935, 1987.