THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 264, No. 14, Issue of May 15, pp. 8052–8058, 1989
Printed in U.S.A.

The Effects of Deletions in the Central Helix of Calmodulin on Enzyme Activation and Peptide Binding*

(Received for publication, August 4, 1988)

Anthony Persechini, Donald K. Blumenthal‡, Harry W. Jarrett§, Claude B. Klee¶, Dianne O. Hardy‖, and Robert H. Kretsinger

From the Department of Biology, University of Virginia, Charlottesville, Virginia 22901, the ‡Department of Biochemistry, University of Texas Health Center, Tyler, Texas 75710, the §Department of Biology, Purdue University School of Science, Indianapolis, Indiana 46223, ¶Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014, and the ‖James Buchanan Brady Urological Institute Research Laboratories, The Johns Hopkins Hospital, Baltimore, Maryland 21205

Using site-directed mutagenesis we have expressed in *Escherichia coli* three engineered calmodulins (CaM) containing deletions in the solvent-exposed region of the central helix. These are CaMΔ84, Glu-84 removed; CaMΔ83–84, Glu-83 and Glu-84 removed; and CaMΔ81–84, Ser-81 through Glu-84 removed. The abilities of these proteins to activate skeletal muscle myosin light chain kinase, plant NAD kinase, and bovine brain calcineurin activities were determined, as were their abilities to bind a synthetic peptide based on the calmodulin-binding domain of skeletal muscle myosin light chain kinase. Similar results were obtained with all three deletion proteins. V_m values for enzymes activated by the deletion proteins are all within 10–20% of those values obtained with bacterial control calmodulin. Relative to bacterial control values, changes in K_{act} or K_d values associated with the deletions are all less than an order of magnitude: K_{act} values for NAD kinase and myosin light chain kinase are increased 5–7-fold, K_d values for binding of the synthetic peptide are increased 4–7-fold, and K_{act} values for calcineurin are increased only 1–3-fold. In assays of NAD kinase and myosin light chain kinase activation some differences between bovine calmodulin and bacterial control calmodulin were observed. With NAD kinase, K_{act} values for the bacterial control protein are increased 4-fold relative to values for bovine calmodulin, and V_m values are increased by 50%; with myosin light chain kinase, K_{act} values are increased 2-fold and V_m values are decreased 10–15% relative to those values obtained with bovine calmodulin. These differences between bacterial control and bovine calmodulins probably can be attributed to known differences in postranslational processing of calmodulin in bacterial and eucaryotic cells. No differences between bovine and control calmodulins were observed in assays of calcineurin activation or peptide binding. Our observations indicate that contacts with the deleted residues, Ser-81 through Glu-84, are not critical in the calmodulin-target complexes we have evaluated. Formation of these calmodulin-target complexes also does not appear to be greatly affected by the global alterations in the structure of calmodulin that are associated with the deletions. In models in which the central helix is maintained in the altered calmodulins, each deleted residue causes the two lobes of calmodulin to be twisted 100° relative to one another and brought 1.5 Å closer together. However, in assays of enzyme activation and peptide binding we have observed little or no difference between CaMΔ84 and either CaMΔ83–84 or CaMΔ81–84. Given a requirement for both lobes of calmodulin in the calmodulin-target complex, then the central helix must bend in order to compensate for effects of the deletions on the relative position of the lobes. This suggests that bending of the central helix also can occur in the native calmodulin-target complex.

Calmodulin is found in all eucaryotic cells, where it regulates in a Ca^{2+}-dependent manner the activities of numerous cellular enzymes (Manalan and Klee, 1984). As seen in the crystal structure (Babu *et al.*, 1985; Kretsinger *et al.*, 1986), it is 65 Å long, with an overall dumbbell shape. The lobes at each end of the dumbbell are both comprised of a pair of EF-hands. The most striking aspect of the structure is the 40 Å central helix, which is shared by the two lobes. The central third of this helix, encompassing residues Arg-74 through Glu-84, bridges the lobes and is entirely exposed to solvent. As defined by Kretsinger and Nockolds (1973) and Kretsinger *et al.* (1986), the central helix of calmodulin contains the "F" helix of the second EF-hand domain in calmodulin on one side of the solvent-exposed region and the "E" helix of the third EF-hand domain on the other. The crystal structure indicates no interactions between the lobes, and various solution studies also show no evidence of significant contacts between them (Andersson *et al.*, 1983; Heidorn and Trewhella, 1988; Seaton *et al.*, 1985; Thulin *et al.*, 1984). However, a disulfide bridge can form between the lobes of an engineered calmodulin that contains cysteine substitutions at positions 3 and 146, which are 37 Å apart in the crystal structure (Persechini and Kretsinger, 1988b). This indicates that in solution the central helix must be flexible, probably in the solvent-exposed region.

We are currently investigating structure-function relations in calmodulin and have focused on the role of the central helix. In assays of enzyme activation and peptide binding, we have evaluated the properties of three altered calmodulins

* This work was supported in part by Grants IN-149D and JFRA-191 from the American Cancer Society (to A. P.), Grants DMB-8608878 (to R. H. K.) and DMB-8702013 from the National Science Foundation (to H. W. J.), Grant GM-39290 from the Public Health Service (to D. K. B.), and grants from the American Heart Association (Texas Affiliate) and the Muscular Dystrophy Association (to D. K. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04729.

that contain deletions of Glu-84, Glu-83 and Glu-84, or Ser-81 through Glu-84, all within the solvent-exposed region of the central helix. Relative to the control calmodulin, all the deletions have similar effects, which amount to increases of less than an order of magnitude in K_d or K_{act} values and changes of less than 20% in V_m values.

In addition to eliminating specific side chains, the deletions we have introduced would be expected to alter the relative position of two lobes, which are on either side of the deleted regions. Our results demonstrate that with the targets we have evaluated there is a notable lack of sensitivity to these changes in the structure of calmodulin. This result is consistent with the model calmodulin-target complex proposed by Persechini and Kretsinger (1988a, 1988b), in which the solvent-exposed region in the central helix of calmodulin functions as a flexible tether, and most contacts are between residues of the target and those in the lobes of calmodulin.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Myosin light chain kinase was isolated from rabbit skeletal muscle as described by Takio *et al.* (1985), calcineurin was isolated from bovine brain as described by Klee *et al.* (1983), and NAD kinase was isolated from pea seedlings as described by Harmon *et al.* (1984). Calmodulins expressed in *Escherichia coli* were isolated essentially as described by Craig *et al.* (1987). The following procedure is for a liter of bacterial culture. 50 ml of media (Bacto-yeast tryptone, 16 g/liter; Bacto-yeast extract, 10 g/liter; and NaCl 5 g/liter; final pH 7.5) containing 25 μg/ml ampicillin is inoculated with a scrape from a frozen stock of bacteria transformed with the desired calmodulin-encoding pKK233-2 construction. Details of cloning procedures are discussed under "Site-directed Mutagenesis." After growth overnight at 37 °C, 25 ml of this culture is used to inoculate a liter of fresh media at 37 °C. After the OD_{550} of this fresh culture has reached a value of 0.3, 300 mM isopropyl-β-D-thiogalactoside is added to a final concentration of 1 mM, followed by growth for an additional 3 h. Bacterial cells are collected by centrifugation at 4 °C and 2500 × *g*, resuspended in 200 ml of ice-cold 50 mM Tris-HCl, pH 7.5, and resedimented at 2500 × *g*. Pelleted cells are suspended in 50 ml of ice-cold lysis buffer that contains 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1 mM DTT.[1] Egg white lysozyme is then added to a final concentration of 200 μg/ml, followed by incubation on ice for 40 min. The bacterial lysate is sonicated with a Branson probe sonicator at a power setting of 70 watts, until the viscosity has dropped sufficiently to allow subsequent handling. Cellular debris is sedimented by centrifugation at 27,000 × *g* for 20 min. The supernatant fraction is removed, and $CaCl_2$ is added to a final concentration of 5 mM. 3 ml of phenyl-Sepharose equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, and 0.1 mM $CaCl_2$) is then added. This mixture is stirred at room temperature for 40 min and washed twice with 10–20 volumes of buffer A. The resin is poured into a column and washed further with buffer A at a flow rate of 50 ml/h until the eluate has an absorbance at 280 nm of less than 0.04. The resin is then washed with buffer A plus 0.5 M NaCl until the absorbance of the eluate is undetectable. Bound calmodulin is eluted with a buffer containing 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, and 1 mM EDTA. Inclusion of NaCl inhibits elution of two minor high molecular weight contaminants. All chromatography procedures are performed at room temperature. Purity of eluted material is characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a protein load of 15 μg followed by Coomassie Blue staining. If protein bands other than calmodulin are detected, a second step of phenyl-Sepharose chromatography is performed. After this second step calmodulins are always electrophoretically homogeneous. Pooled calmodulin fractions are dialyzed exhaustively against 10 mM ammonium bicarbonate and stored frozen at −80 °C. We typically obtain 2–3 mg of pure calmodulin/liter of bacterial culture. Protein concentrations were determined by the Bradford (1976) dye binding assay using a bovine serum albumin protein standard. Calmodulin concentrations were determined from the absorbance at 280 nm using an $E^{0.1\%} = 0.21$ and/or from amino acid compositions. Values determined by these two methods agree to within 5%. Amino acid compositions were determined as described by Jarrett *et al.* (1986), or as described by Bidlingmeyer *et al.* (1984), and Heinrickson and Meredith (1984). N-terminal analysis was performed essentially as described by Hewick *et al.* (1981) and Hunkapillar *et al.* (1984) with an Applied Biosystems 470A protein sequencer. Sodium dodecyl sulfate gel electrophoresis was performed using 15% polyacrylamide slabs as described by Laemmli (1970). Urea gel electrophoresis was performed in the presence of 0.1 mM $CaCl_2$ as described by Persechini *et al.* (1986). Sample mixtures contained 6 M urea, 100 mM Tris-HCl, pH 7.4, 0.1 mM added $CaCl_2$, and various amounts of added calmodulin and melittin (Sigma). Melittin concentrations were calculated from the absorbance at 280 nm, using the extinction coefficient for tryptophan.

Enzyme Assays—Myosin light chain kinase was assayed essentially as described by Blumenthal and Stull (1980), except that a synthetic peptide substrate, KKRPQRATSNYFS-amide, was used instead of mixed myosin light chains. Each reaction mixture contained 0.5 mM peptide substrate, 50 mM MOPS, pH 7.0, 100 μM $CaCl_2$, 4 nM rabbit skeletal muscle myosin light chain kinase, 0.35 mM [γ-^{32}P]ATP (160–300 cpm/pmol), and the indicated total concentrations of calmodulin. Reactions were terminated by applying a 20-μl aliquot to P-81 filter paper followed by immersion in 75 mM phosphoric acid. Subsequent processing of samples was performed as described by Roskowski (1983). Values for K_{act} and V_m were determined by fitting enzyme activation data to a form of the true rate equation (Cha, 1970) with a Marquardt-Levenberg algorithm for a weighted nonlinear parameter estimation using MathView Professional (BrainPower Inc.) software run on a Macintosh computer.

Calcineurin was assayed essentially as described by Manalan and Klee (1983), except that a phosphorylated synthetic peptide was used as substrate. The peptide, which represents a phosphorylation site on the regulatory subunit of cAMP-dependent protein kinase (Blumenthal *et al.*, 1986), was obtained from Peninsula and purified by high performance liquid chromatography on a C-18 column with a gradient of CH_3CN in 0.1% trifluoroacetic acid. Assays were performed at 30 °C for 25 min. Assay mixtures contained 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.5 mM DTT, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, 5 nM calcineurin, and 0.34 μM ^{32}P-phosphorylated peptide (3600 cpm/pmol). Values for K_{act} and V_m were determined from the best fit of the data by a nonlinear regression analysis to a rate equation derived by Richards and Vithayathil (1959).

Plant NAD kinase was assayed as described by Harmon *et al.* (1984). Assays contained 0.29 μg/ml of enzyme and various amounts of added calmodulin. Typically, 13 dilutions of each calmodulin, spanning at least a 100-fold range in concentration, were prepared and assayed. Data for enzyme activation were fit by a nonlinear least squares analysis to the Michaelis-Menten equation, as described previously (Harmon *et al.*, 1984).

Fluorescence Measurements—Fluorescence anisotropy data were measured using an SLM 8000C spectrofluorometer in a "T" configuration. The sample was excited at 370 nm (16 nm bandwidth), and emitted light was passed through Schott KV470 filters. The monochromator of the A channel (vertically polarized) was "removed" by setting it to 0 nm (16 nm slits), whereas the B channel (horizontally polarized) had no monochromator in the light path. Anisotropy values were calculated with an on-line IBM PC using a single-point anisotropy program provided by SLM. Each calmodulin titration experiment was performed at 30 °C in a thermostatted 1-cm cuvette containing 3 M guanidine HCl, 50 mM MOPS, pH 7.0, 1 mM $CaCl_2$, and 6 nM acrylodan-labeled synthetic peptide. Fluorescence emission spectra were collected with the SLM 8000C in ratio mode and the emission monochromator set at 370 nm (8 nm slits). The emission monochromator was scanned between 400 and 600 nm at 2-nm intervals (2 s/data point). The synthetic peptide, KRRWKKAFIAVSAAARFKKC, is based on the calmodulin-binding domain of skeletal muscle myosin light chain kinase (Blumenthal *et al.*, 1985). It was labeled by overnight reaction with a 2-fold molar excess of acrylodan (Molecular Probes, Inc.) at room temperature in 20% acetonitrile, 80% H_2O (v/v), 5 mM MOPS, pH 7.0. Labeled peptide was purified by reversed-phase high performance liquid chromatography. The molar extinction coefficient measured at 365 nm indicated that 1 mol of reagent was incorporated/mol of labeled peptide.

Fluorescence anisotropy data were analyzed to determine K_d values of peptide binding to calmodulin by using weighted nonlinear parameter estimation and by using the linear Scatchard analysis. These two

[1] The abbreviations used are: DTT, dithiothreitol; CaM, calmodulin; CaMΔ84, an engineered calmodulin in which Glu-84 has been deleted; CaMΔ83–84, Glu-83 and Glu-84 deleted; CaMΔ81–84, Ser-81 through Glu-84 deleted; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

techniques gave estimates of K_d that were in good agreement with each other. Equations relating observed anisotropies to fractional saturations are based on the original derivations of Evett and Isenberg (1969) and Weber (1952). In the case of the nonlinear curve-fitting technique, the data from each titration experiment were fit to the equation:

$$r_{obs} = (Z(r_{max} + r_{min})/(1 + Z)$$

where

$$Z = (F_{max}/F_{min})/\{1/((1/Y) - 1)\}$$

and

$$Y = \{K_d + [CaM]_{tot} + [peptide]_{tot} - ((K_d + [CaM]_{tot} + [peptide]_{tot})^2 - 4[CaM]_{tot}[peptide]_{tot})^{0.5}\}/2[peptide]_{tot}$$

The expression for Y, the fraction of calmodulin bound to the peptide, is in the form of the "true-rate" equation (Cha, 1970). The Marquardt-Levenberg algorithm for nonlinear parameter estimation included in the MathView Professional (BrainPower, Inc.) software package for the Macintosh computer was used for fitting. Each mean value of observed anisotropy (r_{obs}) was weighted using the standard deviation determined for that set of observations. For Scatchard analysis, the concentration of bound calmodulin, B, was calculated using the expression:

$$B = [peptide]_{tot}/\{(F_{max}/F_{min})((r_{max} - r_{obs})/(r_{obs} - r_{min})) + 1\}$$

and the concentration of free calmodulin, F, calculated as:

$$F = [CaM]_{tot} - B$$

The K_d value for each calmodulin titration was determined from the slopes of Scatchard plots (B *versus* B/F) after fitting the data to a line using unweighted least squares linear regression analysis. The value of r_{max}, the anisotropy observed at saturating concentrations of a given calmodulin, was calculated from the mean of the r_{obs} values taken at calmodulin concentrations in excess of 200 nM; these values were: 0.1488 (bovine); 0.1474 (control); 0.1437 (CaMΔ84); 0.1339 (CaMΔ83–84); 0.1325 (CaMΔ81–84). The value of r_{min}, the anisotropy of the peptide in the absence of calmodulin, used in all calculations was 0.0827. The value of F_{max}/F_{min}, the ratio of the fluorescence intensity of the calmodulin-bound peptide relative to that of the free peptide, was determined using bovine calmodulin under instrument conditions identical to those described above for the anisotropy measurements. These particular measurements were made by setting the excitation polarizer to vertical and the emission polarizer to 54.7°, in order to eliminate polarization effects on fluorescence intensity. After correcting for blank, the value of F_{max}/F_{min} was determined to be 1.68. Since the emission spectra for all the calmodulin-peptide complexes used in this study were virtually identical, this value for F_{max}/F_{min} was used for all calculations. The terms $[CaM]_{tot}$ and $[peptide]_{tot}$ represent the total concentrations of calmodulin and acrylodan peptide, respectively.

Site-directed Mutagenesis—A partial calmodulin-encoding cDNA was isolated from an *Arbacia punctulata* λgt11 library as described by Hardy *et al.* (1988). Site-directed mutagenesis was performed to create an *Nco*I site at the natural ATG start codon and a missing region, encoding the last seven amino acids, was produced synthetically. The sequence of the final M13mp18 (Norrander *et al.*, 1983) clone is shown in Fig. 1. Some 5′-untranslated sequence is maintained in this clone. A protein identical to vetebrate calmodulin is encoded. Site-directed changes in the calmodulin-encoding sequence were introduced using mismatched primer extension mutagenesis of M13mp18 single-stranded DNA as described by Zoller and Smith (1983), except duplex DNA was not enriched after *in vitro* primer extension. The lengths of mismatched primers were between 21 and 23 bases, with mismatches located in approximately the center of the primer. Phage containing the desired changes were identified by differential hybridization, using the [γ-^{32}P]ATP-phosphorylated mismatched primer as a probe, and plaque-purified (Watterson *et al.*, 1980). For expression experiments, desired calmodulin-encoding sequences were excised from M13mp18 plasmid by cleaving with *Nco*I and *Pst*I restriction endonucleases. The resulting fragments were cloned in the expression vector pKK233-2 (Pharmacia LKB Biotechnology, Inc.) opened by *Nco*I and *Pst*I restriction digestion. Stocks of bacteria transformed with calmodulin expression vectors were grown from isolated ampicillin-resistant colonies and stored in 40% glycerol at −80 °C. All calmodulin-encoding cDNAs were sequenced (Biggin *et al.*, 1981; Sanger and Coulsen, 1978) in their entirety before being expressed. Routine cloning procedures were performed as described by Maniatis *et al.* (1982). *E. coli* JM101 was the host strain used for all procedures.

Molecular Modeling—Molecular modeling was performed with the calmodulin coordinates available in our laboratory, where the crystal structure is currently undergoing refinement. The UCSD Molecular Modeling System run on an Iris 4040 was used to display and manipulate structures.

RESULTS

Modified and Control Bacterial Calmodulins—The sequence of the cDNA template encoding control bacterial calmodulin is presented in Fig. 1. Using primer-extension mutagenesis techniques (Zoller and Smith, 1983), we made three deletions in the region of the template encoding residues 81–84. The encoded deletion proteins are termed CaMΔ84, Glu-84 deleted; CaMΔ83–84, Glu-83 and Glu-84 deleted; and CaMΔ81–84, Ser-81 through Glu-84 deleted. The identity of bacterially expressed proteins was confirmed by amino acid analysis (data not shown). An unblocked alanine was found at the N terminus of the bacterial protein, in agreement with the results of Putkey *et al.* (1985) and Roberts *et al.* (1985). This is different from the bovine protein, in which the N terminus is acetylated (Watterson *et al.* 1980). It has been shown that calmodulin expressed in *E. coli* lacks trimethyllysine, which

```
1          11         21         31         41         51
AGTCGTTCTGATCTCATCAGCACTTCGACATCGATCCTGCTTTCGACACGTGTCTGTAGC

61         71         81         91         101        111
CACAAACACAATTCGTTCCAGTCGTGCAAATATCTAGCGACCCTACTGCTGCCCAAAAAC

                                A  D  Q  L  T  E  E  Q  I  A  E
121        131        141        151        161        171
TTGTTGACGTTTCGCAACTGCGACCATGGCTGACCAACTCACCGAGGAACAGATTGCAGA
                          NcoI

  F  K  E  A  F  S  L  F  D  K  D  G  D  G  T  I  T  T  K  E
181        191        201        211        221        231
ATTCAAGGAAGCTTTCTCCCTGTTCGACAAGGACGGCGATGGCACAATCACAACCAAGGA

  L  G  T  V  M  R  S  L  G  Q  N  P  T  E  A  E  L  Q  D  M
241        251        261        271        281        291
GCTGGGGACCGTGATGAGATCTCTTGGTCAGAACCCGACAGAGGCTGAGCTCCAAGACAT

  I  N  E  V  D  A  D  G  N  G  T  I  D  F  P  E  F  L  T  M
301        311        321        331        341        351
GATCAACGAGGTCGATGCCGATGGAAATGGGACCATAGATTTCCCAGAGTTCCTCACGAT

  M  A  R  K  M  K  D  T  D  S[81] E  E  E[84] I  R  E  A  F  R  V
361        371        381        391        401        411
GATGGCGAGGAAGATGAAGGACACGGACAGCGAAGAGGAGATCAGGGAAGCCTTCAGAGT

  F  D  K  D  G  N  G  Y  I  S  A  A  E  L  R  H  V  M  T  N
421        431        441        451        461        471
CTTTGACAAGGATGGCAACGGCTACATCAGCGCTGCTGAGCTACGTCACGTGATGACGAA

  L  G  E  K  L  T  D  E  E  V  D  E  M  I  R  E  A  D  I  D
481        491        501        511        521        531
TCTCGGCGAGAAGCTGACAGACGAGGAGGTGGACGAAATGATCAGGGAAGCCGACATCGA

  G  D  G  Q  V  N  Y  E  E  F  V  Q  M  M  T  A  K
541        551        561        571        581        591
TGGTGACGGACAGGTCAACTATGAAGAATTTGTGCAAATGATGACGGCTAAGTGACTGCAG
                                                    PstI
```

FIG. 1. **The sequence of calmodulin-encoding cDNA cloned in M13mp18.** *Xmn*I and *Pst*I ends were used to clone this sequence into an M13mp18 vector cut with *Sma*I and *Pst*I restriction endonucleases. The 3′-terminal *Pst*I site is indicated in the figure. The *Nco*I site used to clone the calmodulin-encoding segment into a pKK233-2 expression vector is also indicated. The ATG translation start and TGA translation stop sites are shown in *boldface*. The calmodulin sequence is shown above the DNA sequence encoding it. The sequence encoding residues Ser-81 through Glu-84 is also shown in *boldface*. Deletions in this region result in the following new sequences: GAC**AGCGAAGAG**ATC, CaMΔ84; GAC**AGCGAA**-ATC, CaMΔ83–84; GATATC, CaMΔ81–84. Note that the sequence encoding Asp-80 in CaMΔ81–84 has been changed from GAC to GAT, so as to create a new *Eco*RV site.

is found in the native protein at position 115 (Putkey *et al.*, 1985; Roberts *et al.*, 1985). We have not determined the trimethyllysine content in our calmodulin preparations, but assume that Lys-115 is not methylated.

The electrophoretic mobilities on urea polyacrylamide gels of bovine and bacterial calmodulins in the presence and absence of melittin are seen in Fig. 2. Melittin and related peptides are known to form a complex with calmodulin that is stable in urea and has an electrophoretic mobility that is slower than that of the uncomplexed protein (Comte *et al.*, 1983). In the absence of melittin a small progressive decrease in relative mobility is seen with the deletion proteins; this appears to be proportional to the number of deleted glutamic acid residues. With the more slowly migrating calmodulin-melittin complexes the effects of the deletions on relative mobilities are more pronounced. These differences in electrophoretic mobilities among the bacterial calmodulins are probably due to the loss of negative charge associated with successive deletions of glutamic acid. They are probably enhanced in the complex with a basic melittin peptide because of charge neutralization. A slightly decreased mobility relative to bovine calmodulin is seen with the control bacterial protein. This is probably due to the lack of acetylation at the N terminus and/or trimethylation of Lys-115. On sodium dodecyl sulfate polyacrylamide gels the electrophoretic mobilities of bovine calmodulin and the bacterial control and deletion proteins are not significantly different (not shown).

Enzyme Activation—The abilities of bovine and bacterial calmodulins to elevate skeletal muscle myosin light chain kinase, pea seedling NAD kinase, and bovine brain calcineurin activities were compared. Data for enzyme activation by the bacterial calmodulins are seen in Figs. 3 and 4. Typical values for K_{act}, the concentration of calmodulin giving half-maximal activation, and V_m are presented in Table I. For each of the enzyme activities assayed, all three deletion proteins have similar activation kinetics, which differ from what is observed with bacterial control calmodulin. With plant NAD kinase and myosin light chain kinase, K_{act} values are increased 5–7-fold relative to the bacterial control. With these two enzymes, a trend toward a reduced V_m value appears to be associated with increasing deletion size. These changes in V_m values are 10% or less, and we have not yet established their significance. K_{act} values for activation of calcineurin by the deletion proteins are 2–3-fold greater than bacterial control values, and V_m values are consistently 15–20% lower. As seen in Fig. 3*A*,

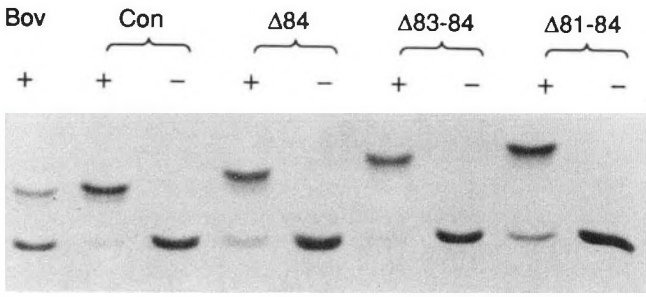

FIG. 2. **The effect of melittin on the electrophoretic mobilities of bovine, control, and deletion calmodulins in urea polyacrylamide gels.** Samples were prepared as described under "Experimental Procedures" in the presence (+) and absence (−) of added melittin. For bovine calmodulin, electrophoresis was performed only in the presence of melittin at a molar ratio to calmodulin of 0.4. For the bacterial calmodulins mellitin was added in a molar ratio of 0.7 to 0.9 with respect to calmodulin, so in some + lanes protein bands corresponding to both free calmodulin and the calmodulin-melittin complex are visible. *Bov*, bovine calmodulin; *Con*, bacterial control; *Δ84*, CaMΔ84; *Δ83–84*, CaMΔ83–84; *Δ81–84*, CaMΔ81–84.

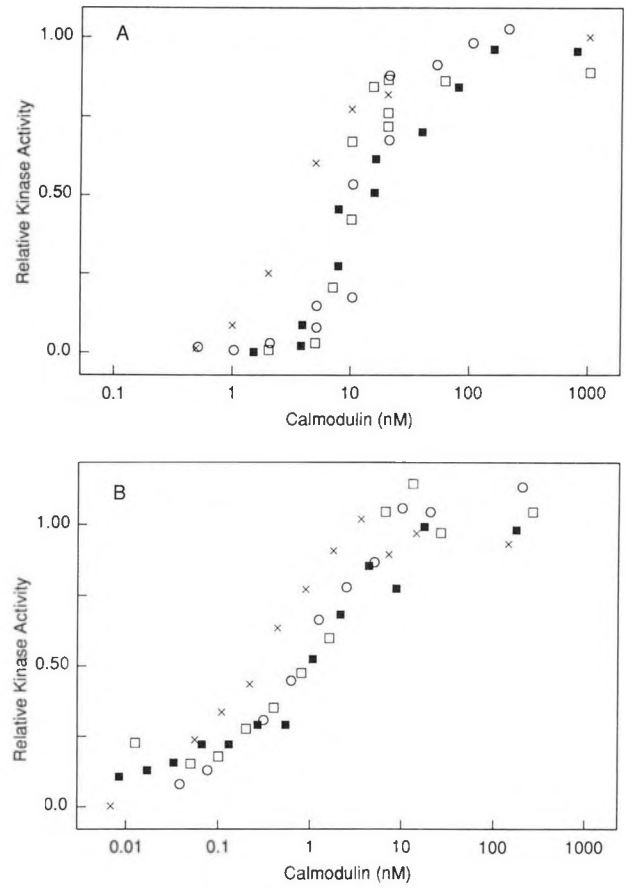

FIG. 3. **Activation of (*A*) skeletal muscle myosin light chain kinase and (*B*) plant NAD kinase activities by control and deletion calmodulins.** Kinase activity is expressed as a fraction of the maximal level attained with the bacterial control. The indicated calmodulin concentrations are the total amounts added to the assay mixtures. In assays of myosin light chain kinase activity, concentrations of synthetic peptide substrate and enzyme were 0.5 mM and 4 nM, respectively. Plant NAD kinase assay mixtures contained 0.29 μg/ml enzyme. ×, control; ○, CaMΔ84; ■, CaMΔ83–84; □, CaMΔ81–84.

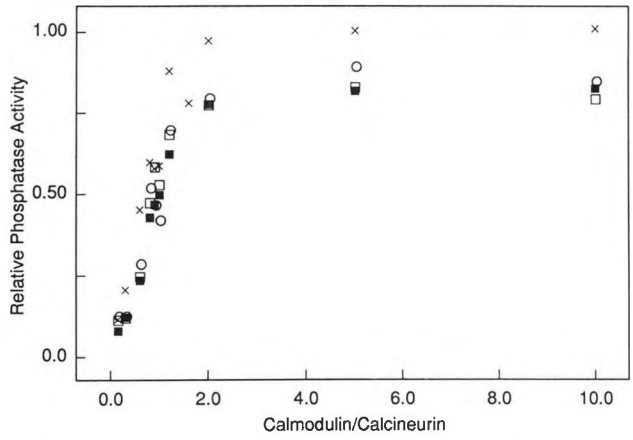

FIG. 4. **Activation of bovine brain calcineurin by control and deletion calmodulins.** Fractional activity is expressed relative to the maximal level of activation obtained with the bacterial control calmodulin. The ratio, calmodulin/calcineurin, is the molar ratio of added calmodulin to calcineurin in the assay mixture. Concentrations of phosphorylated peptide substrate and calcineurin were 0.34 μM and 5 nM, respectively. *Symbols* defined as in Fig. 3.

TABLE I

Steady state kinetic parameters for activation of various calmodulin-dependent enzymes by bovine, control, and deletion calmodulins

V_m values are expressed as a fraction of the V_m value determined with bovine calmodulin. K_{act} is the concentration of calmodulin required for half-maximal enzyme activation. Values for K_{act} and V_m were estimated from enzyme kinetic data as described under "Experimental Procedures." For all three enzyme activities, V_m values determined from duplicate data sets were seen to vary by less than 10%. Duplicate determinations of K_{act} values for myosin light chain kinase and plant NAD kinase were within a factor of two; calcineurin K_{act} values are ± 50%.

Calmodulin	K_{act}	V_m (fractional)
	nM	
Myosin light chain kinase activation		
Bovine	1.2	1.0
Control	2.4	0.90
CaMΔ84	12	0.90
CaMΔ83–84	14	0.83
CaMΔ81–84	7.9	0.78
NAD kinase activation		
Bovine	0.06	1.0
Control	0.27	1.55
CaMΔ84	1.07	1.77
CaMΔ83–84	1.87	1.60
CaMΔ81–84	1.65	1.47
Calcineurin activation		
Bovine	0.25	1.0
Control	0.3	1.01
CaMΔ84	0.75	0.87
CaMΔ83–84	0.6	0.81
CaMΔ81–84	0.75	0.82

curves for myosin light chain kinase activation by the deletion proteins are all somewhat steeper than the curve for activation by the bacterial control. The reason for this difference is not known.

There are no apparent differences between bovine and control bacterial calmodulins with regard to calcineurin activation, but differences are seen with the other two enzymes. With NAD kinase K_{act} values for the bacterial control are increased 4-fold relative to values for bovine calmodulin, and V_m values are increased by 50–80%. With myosin light chain kinase there is a 2-fold increase in K_{act} values and a consistent 10–15% reduction in V_m values. Roberts *et al.* (1985) have also reported an increase in V_m values for activation of NAD kinase by bacterial calmodulin. They attribute this to the lack of methylation at Lys-115 in the bacterial protein (Roberts *et al.*, 1985). The other smaller differences between bacterial control and bovine calmodulins that we report here probably are also due to the lack of trimethyllysine-115 and/or *N*-acetylation in the bacterial proteins.

Peptide Binding—We have synthesized a peptide based on the calmodulin-binding domain of skeletal muscle myosin light chain kinase (Blumenthal *et al.*, 1985). This peptide has the sequence: K^{1}-R-R-W-K-K-A-F-I-A^{10}-V-S-A-A-A-R-F-K-K-C^{20}. We have stoichiometrically labeled Cys-20 with a fluorescent molecule, acrylodan, as described under "Experimental Procedures." We have evaluated by fluorescence spectroscopy the complexes between labeled peptide and bovine or the various bacterial calmodulins. No significant differences were observed in the acrylodan fluorescence emission spectra of the various complexes, whose maxima range from 504 to 508 nm. Typical uncorrected emission spectra for peptides in the presence and absence of saturating bovine calmodulin are presented in Fig. 5. We have estimated K_d values for the calmodulin-peptide complexes by monitoring calmodulin-dependent changes in acrylodan fluorescence anisotropy. Mean values (10 determinations per data point) for acrylodan fluo-

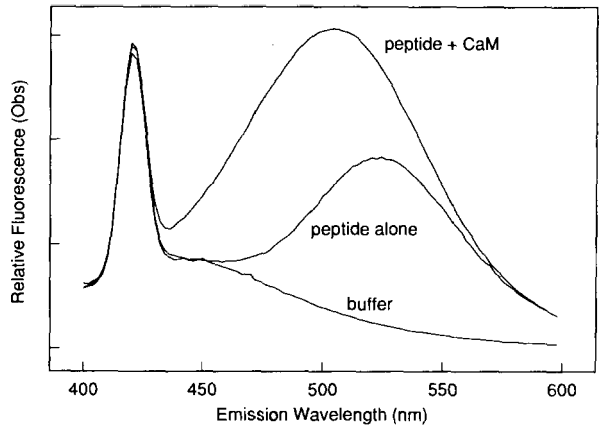

FIG. 5. **Fluorescence emission spectra for acrylodan-labeled peptide in the presence and absence of a saturating level of bovine calmodulin.** Samples were excited at a wavelength of 370 nm, and emission spectra were measured as described under "Experimental Procedures." Added bovine calmodulin (*CaM*) was 10-fold in excess over the 20 nM peptide concentration. The apparent emission spectrum for buffer alone is also shown in the figure. All spectra are uncorrected.

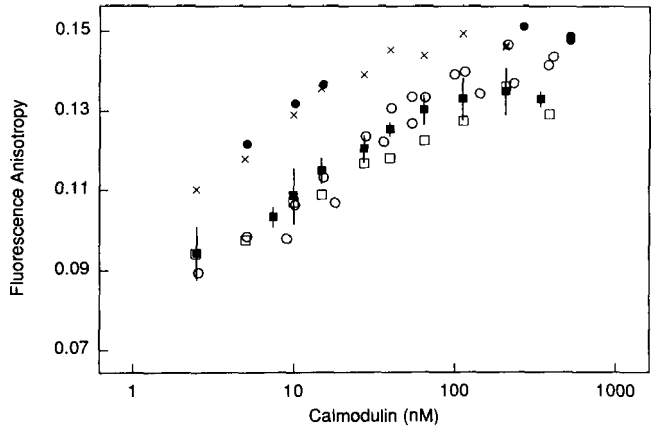

FIG. 6. **Acrylodan-labeled peptide fluorescence anisotropy measured at various concentrations of added bovine or bacterial calmodulins.** Each point represents the mean of 10 determinations. Standard deviations for the CaMΔ81–84 data set are also shown. Samples were excited at 370 nm, and the peptide concentration in each experiment was 6 nM. Further experimental details are presented under "Experimental Procedures." ●, bovine calmodulin; ×, control; ○, CaMΔ84; ■, CaMΔ83–84; □, CaMΔ81–84.

TABLE II

Binding of the acrylodan-labeled peptide by bovine, control, and deletion calmodulins

K_d values calculated from duplicate data sets were seen to vary by less than a factor of 2. Values for the acrylodan fluorescence emission maxima, $Emission_{max}$, in the various complexes are also shown. Further details of fitting procedures, peptide structure, and fluorescence measurements are presented under "Experimental Procedures."

Calmodulin	K_d[a]	K_d[b]	$Emission_{max}$
	nM		*nm*
Bovine	3.7	3.2	504
Control	3.9	4.0	506
CaMΔ84	25	32	504
CaMΔ83–84	13	12	508
CaMΔ81–84	17	18	508

[a] Calculated using a weighted nonlinear curve-fitting procedure.
[b] Determined from Scatchard plots (not shown).

rescence anisotropy measured at various concentrations of added bovine or bacterial calmodulin are presented in Fig. 6. Standard deviations for the CaMΔ83–84 data set are also shown. Similar standard deviations were observed for the other calmodulin data sets but were not included in order to improve the clarity of the figure. As seen in Table II, calculated K_d values for bovine and control calmodulins are not significantly different, and all three deletions within the central helix cause a 4–7-fold increase in K_d values. Similar differences were seen in K_d values estimated from calmodulin-dependent changes in acrylodan fluorescence intensity, measured at 490 nm (not shown).

DISCUSSION

Our observations indicate that residues Ser-81 through Glu-84 can be deleted from the solvent-exposed region in the central helix without greatly affecting calmodulin's ability to interact with the targets we have evaluated. Relative to bacterial control values, K_{act} values for NAD kinase and myosin light chain kinase are increased 5–7-fold, K_d values for binding of the peptide are increased 4–7-fold, and K_{act} values for calcineurin are increased only 2–3-fold. All these differences in K_{act} or K_d values are less than 1 order of magnitude, and V_m values for enzymes activated by the deletion proteins are all within 10–20% of control values. The parallel effects of the deletions on binding of skeletal muscle myosin light chain kinase and the synthetic peptide derived from its sequence support the previous localization of the calmodulin-binding domain in the kinase to a short peptide (Blumenthal *et al.*, 1985) represented by the synthetic one used in our studies.

Craig *et al.* (1987) have replaced the three glutamic acids encompassing residues 82–84 of the central helix with three lysines. This altered calmodulin is able to activate fully calmodulin-dependent cAMP phosphodiesterase activity, but it can elevate myosin light chain kinase activity to only 30% of the maximal level seen with a control protein and fails to activate NAD kinase activity. K_{act} values for both myosin light chain kinase and phosphodiesterase are increased about 2-fold relative to control values. Our results indicate that these effects of lysine substitutions reported by Craig *et al.* (1987) probably are not due to the elimination of contacts with residues in the glutamic acid cluster.

Interpretation of our results requires consideration of two very different levels at which the deletions affect calmodulin structure. The first is at the local level and involves alterations in specific side chain interactions in the region of the deleted residues. The second is at the global level and involves changes in the relative positions of the two calmodulin lobes. This latter effect arises because, as illustrated in Fig. 7, the deletions are located in the solvent-exposed region of the central helix, where it bridges the two lobes. In a model in which the central helix is maintained, each deleted residue causes the two lobes of calmodulin to be twisted 100° relative to one another and brought 1.5 Å closer together. In the functional assays we have performed there is little or no difference between CaMΔ84, which contains only a single amino acid deletion, and either CaMΔ83–84 or CaMΔ81–84. Therefore, the major effect of the deletions on calmodulin function can be attributed to removal of a single glutamic acid from the cluster: E^{82}-E-E^{84}. One of these residues may form a stabilizing electrostatic hydrogen bond with a residue in the target or in calmodulin itself. The presumed global structural changes associated with the deletions appear to have little effect on calmodulin's ability to bind or activate the targets we have evaluated. If both lobes of calmodulin are required for formation of complexes with these targets, then this means that

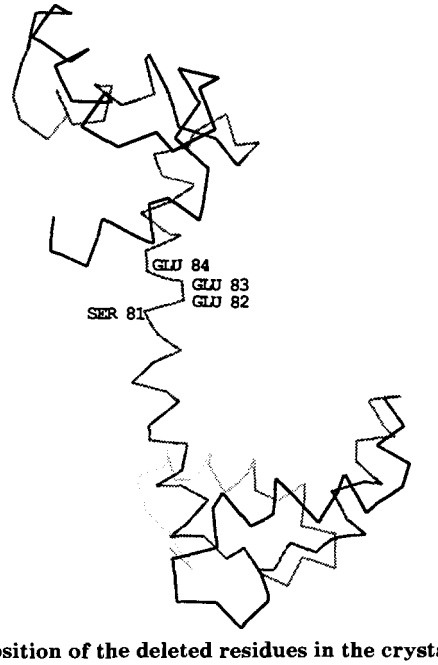

FIG. 7. **Position of the deleted residues in the crystal structure of calmodulin.** An α-carbon tracing of the calmodulin crystal structure, which is currently being refined in our laboratory, is presented. The C-terminal lobe of calmodulin is uppermost, and the positions in the central helix of residues Ser-81 through Glu-84 are indicated.

in the calmodulin-target complex the lobes of all three deletion proteins can adopt similar relative positions. This clearly requires some bending of the central helix, which would otherwise hold the lobes of each deletion protein in a different relative orientation. It is possible that targets interact with only one of calmodulin's two lobes, so their relative positions are not important. While this cannot yet be ruled out with NAD kinase, published data suggest that interactions with several targets, including myosin light chain kinase, cAMP phosphodiesterase, and calcineurin, involve both lobes of calmodulin. If the two lobes are separated by trypsinolysis in the solvent-exposed region of central helix, they cannot activate myosin light chain kinase, cAMP phosphodiesterase, and calcineurin enzyme activities (Newton *et al.*, 1984, 1985). However, if the fragments resulting from trypsinolysis are tethered by a chemical cross-link, their ability to activate enzymes is retained (Persechini and Kretsinger, 1988b). Klevit *et al.* (1985) have demonstrated that residues in both lobes of calmodulin are perturbed in the complex with a synthetic peptide based on the calmodulin-binding domain of skeletal muscle myosin light chain kinase. Furthermore, it is known that activation of both myosin light chain kinase and cAMP phosphodiesterase activities requires occupancy of all four Ca^{2+}-binding sites in calmodulin (Blumenthal and Stull, 1980; Huang *et al.*, 1981).

Our results suggest that the central helix of calmodulin is bent in complexes between one or more of the deletion proteins and the calmodulin targets we have evaluated. Persechini and Kretsinger (1988b) have recently shown that this is probably also true for some native calmodulin-target complexes. They have created a 16 Å bismaleimidohexane cross-link between cysteines substituted in calmodulin at positions 3 and 146, which are 37 Å apart in the crystal structure. Although this undoubtedly creates a bend in the central helix, it does not affect the ability of calmodulin to activate myosin light chain kinase activity. This indicates that in the native calmodulin-myosin light chain kinase complex the central

helix is probably also bent, with the relative position of the two lobes different from that seen in the crystal structure. A model for this type of calmodulin-target complex has been proposed by Persechini and Kretsinger (1988a, 1988b). In their model the solvent-exposed region in the central helix functions as a flexible tether, and most contacts with the target involve residues in the two lobes of calmodulin. Hydrophobic patches associated with each lobe are juxtaposed with hydrophobic patches on the target. Given this type of model calmodulin-target complex, we might expect a degree of adaptability to deletions or insertions within the central helix of calmodulin.

It is now well established that the entire sequence of calmodulin, including that of the central helix, is well conserved among a varied group of eucaryotic organisms, which represent millions of years of evolution (Manalan and Klee, 1984). Yet, we have shown that Ser-81 through Glu-84 can be deleted from the central helix with little effect on the calmodulin-target complexes we have evaluated. The effects that are observed can be attributed mostly to removal of a single glutamic acid, and presumed changes in the relative positions of the two lobes of calmodulin caused by the deletions appear to have minimal consequence on calmodulin function. How can these observations be reconciled with the conservation of primary structure seen in the central helix?

Stimulus-induced changes in intracellular Ca^{2+} ion are transient in nature, hence formation of Ca^{2+}-calmodulin and subsequent activation of its targets are dynamic processes. It is, therefore, difficult to predict how small changes in *in vitro* steady state interactions, such as those we report here, might affect *in vivo* cellular function. Furthermore, we have assayed the abilities of the deletion proteins to activate only three of calmodulins more than 20 biological targets (Manalan and Klee, 1984). We might expect interactions with some of these targets to be more severely affected than others by the changes we have made in the central helix. Indeed, there is good evidence that contacts between calmodulin and its targets vary considerably among the different calmodulin-target complexes (Craig *et al.*, 1987; Manalan and Klee, 1984; Newton *et al.*, 1984, 1985).

While there are undoubtedly unique aspects to the interactions between calmodulin and each of its targets, at a gross level there are essentially three possible classes of calmodulin-target complexes, which can be described in terms of the relative positions of the two lobes of calmodulin. In class I complexes the target interacts with only one lobe of calmodulin or the other, so that the relative positions of the lobes are not important. In class II complexes there are interactions with both lobes of calmodulin, and their relative positions are close to what is found in the crystal structure. In class III complexes there are also interactions with both lobes of calmodulin, but they adopt relative positions different from what is observed in the crystal structure, most likely as a result of bending or distortion within the central helix of calmodulin. The targets we have evaluated in this study probably form class III complexes with calmodulin. However, as discussed elsewhere (Persechini and Kretsinger, 1988a), there are also examples of biological targets that appear to form class I complexes with calmodulin. Further definition of the various classes of calmodulin-target complexes is essential if we are to understand structure-function relations in calmodulin and determine how it orchestrates so many different aspects of cellular function.

Acknowledgments—We would like to thank Martha J. Spano and Theresa Honeycutt for expert technical assistance, and Robert J. Cordaro and Paul S. Yadlowsky for assistance with computer applications. We would also like to acknowledge the Commonwealth of Virginia Protein and Nucleic Acid Sequencing Facility for performing amino acid analysis, protein sequencing, and oligonucleotide synthesis.

REFERENCES

Andersson, A., Forsén, S., Thulin, E., and Vogel, H. J. (1983) *Biochemistry* **22,** 2309–2313
Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) *Nature* **315,** 37–40
Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984) *J. Chromatogr.* **336,** 93–104
Biggin, M. D., Gibson, T. J., and Hong, G. F. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3963–3965
Blumenthal, D. K., and Stull, J. T. (1980) *Biochemistry* **19,** 5608–5614
Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., and Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3187–3191
Blumenthal, D. K., Takio, K., Hansen, R. S., and Krebs, E. G. (1986) *J. Biol. Chem.* **261,** 8140–8145
Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
Cha, S. (1970) *J. Biol. Chem.* **245,** 4814–4818
Comte, M., Maulet, Y., and Cox, J. A. (1983) *Biochem. J.* **209,** 269–272
Craig, T. A., Watterson, D. M., Prendergast, F. G., Haiech, J., and Roberts, D. M. (1987) *J. Biol. Chem.* **262,** 3278–3284
Evett, J., and Isenberg, I. (1969) *Ann. N. Y. Acad. Sci.* **158,** 210–222
Hardy, D. O., Bender, P. K., and Kretsinger, R. H. (1988) *J. Mol. Biol.* **199,** 223–227
Harmon, A. C., Jarrett, H. W., and Cormier, M. J. (1984) *Anal. Biochem.* **141,** 168–178
Heidorn, D. B., and Trewhella, J. (1988) *Biochemistry* **27,** 909–915
Heinrikson, R. L., and Meredith, S. C. (1984) *Anal. Biochem.* **136,** 65–74
Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) *J. Biol. Chem.* **256,** 7990–7997
Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H., and Sharma, R. K. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 871–874
Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., and Hood, L. E. (1984) *Methods Enzymol.* **91,** 399–413
Jarrett, H. W., Cooksy, K. D., Ellis, B., and Anderson, J. M. (1986) *Anal. Biochem.* **153,** 189–198
Klee, C. B., Krinks, M. H., Manalan, A. S., Cohen, P., and Stewart, A. A. (1983) *Methods Enzymol* **102,** 227–244
Klevit, R. E., Blumenthal, D. K., Wemmer, D. E., and Krebs, E. G. (1985) *Biochemistry* **24,** 8152–8157
Kretsinger, R. H., and Nockolds, C. E. (1973) *J. Biol. Chem.* **248,** 3313–3326
Kretsinger, R. H., Rudnick, S. E., and Weissman, L. J. (1986) *J. Inorg. Biochem.* **28,** 289–302
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Manalan, A. S., and Klee, C. B. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 4291–4295
Manalan, A. S., and Klee, C. B. (1984) *Adv. Cyclic Nucleotide Res.* **18,** 227–277
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., and Klee, C. B. (1984) *J. Biol. Chem.* **259,** 4419–4426
Newton, D., Klee, C., Woodget, J., and Cohen, P. (1985) *Biochim. Biophys. Acta* **845,** 533–539
Norrander, J., Kempe, T., and Messing, J. (1983) *Gene (Amst.)* **26,** 101–106
Persechini, A., and Kretsinger, R. H. (1988a) *J. Cardiovasc. Pharmacol.* **12,** Suppl. 5, S1–S12
Persechini, A., and Kretsinger, R. H. (1988b) *J. Biol. Chem.* **263,** 12175–12178
Persechini, A., Kamm, K. E., and Stull, J. T. (1986) *J. Biol. Chem.* **261,** 6293–6299
Putkey, J. A., Slaughter, G. R., and Means, A. R. (1985) *J. Biol. Chem.* **260,** 4704–4712
Richards, F. M., and Vithayathil, P. J. (1959) *J. Biol. Chem.* **234,** 1459–1465
Roberts, D. M., Crea, R., Malecha, M., Alvarado-Urbina, G., Chiarello, R. H., and Watterson, D. M. (1985) *Biochemistry* **24,** 5090–5098
Roskowski, R. (1983) *Methods Enzymol.* **99,** 3–6
Sanger, R., and Coulsen, A. R. (1978) *FEBS Lett.* **87,** 107–110
Seaton, B. A., Head, J. F., Engelman, D. M., and Richards, F. M. (1985) *Biochemistry* **24,** 6740–6743
Takio, K., Blumenthal, D. K., Edelman, A. M., Walsh, K. A., Krebs, E. G., and Titani, K. (1985) *Biochemistry* **24,** 6028–6037
Thulin, E., Andersson, A., Drakenberg, T., Forsén, S., and Vogel, H. J. (1984) *Biochemistry* **23,** 1862–1870
Watterson, D. M., Sharief, F., and Vanaman, T. C. (1980) *J. Biol. Chem.* **255,** 962–975
Weber, G. (1952) *Biochem. J.* **51,** 145–155
Zoller, M. J., and Smith M. (1983) *Methods Enzymol.* **100,** 486–487