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

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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, bacterial 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. Vm 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 Kd or Ka values associated with the deletions are all less than an order of magnitude: Kd values for NAD kinase and myosin light chain kinase are increased 5–7-fold, Kd values for binding of the synthetic peptide are increased 4–7-fold, and Ka 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, Ka values for the bacterial control protein are increased 4-fold relative to values for bovine calmodulin, and Vm values are increased by 50%; with myosin light chain kinase, Ka values are increased 2-fold and Vm 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 posttranslational 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 Ca2+-dependent manner the activities of numerous cellular enzymes (Manalan and Klee, 1984). As seen in the crystal structure (Babu et al., 1985; Kreisenger 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 Kreisenger and Nockolds (1973) and Kreisenger 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., 1988; Heidorn and Trewehella, 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 Kreisenger, 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.
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 $K_v$ or $K_{on}$ 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 Perschini 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. (1983), calcein was isolated from bovine brain as described by Klee et al. (1982).**

Mutants in the calmodulin central helix was from rabbit skeletal muscle as described by Takio et al. (1983), calcein was isolated from bovine brain as described by Klee et al. (1982). The resin was then washed with buffer A plus 0.5 M NaCl until the absorbance of the eluate is undetectable. Bound calmodulin is eluted by setting it to 0 nm (16 nm slits), whereas the B channel (horizontally oriented) was determined from the best fit of the data by a nonlinear regression analysis to a rate equation derived by Richards and Vithayathil (1959).

Fluorescence Measurements—Fluorescence anisotropy data were measured using an SLM 8000C spectrophotometer in a “T” configuration. The sample was excited at 570 nm (16 nm bandwidth), and emitted light was passed through Schott KV470 filters. The monochromator of the B channel (horizontally 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 CaCl₂, and 6 mM 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 peptide, which represents a phosphorylation site on skeletal muscle myosin light chain kinase, 0.34 μM [γ-32P]ATP (160–300 cpm/pmml), and the isolated total concentrations of calmodulin. Reactions were terminated by adding 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 Reskowski (1980). Value of $K_v$ 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).
Deletions in the Calmodulin Central Helix

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

RESULTS
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 those of bacterial control values, and $V_m$ values are consistently 15–20% lower. As seen in Fig. 3A,

![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 in the presence of melittin at a molar ratio to calmodulin of 0.4. For the bacterial calmodulins melittin 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, CaMA84; Δ83-84, CaMA83-84; Δ81-84, CaMA81-84.](attachment:fig2.png)

![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 mM Mg and 0.34 tryptophan. X, control; O, CaMA84; ■, CaMA83-84; □, CaMA81-84.](attachment:fig3.png)

![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.](attachment:fig4.png)
Deletions in the Calmodulin Central Helix

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

| Enzyme Activity          | Calmodulin | \( K_a \) (nM) | \( V_m \) (fractional) |
|--------------------------|------------|----------------|------------------------|
| 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           | 1.65                   |
|                          | 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 \( K_a \) 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 \( K_a \) 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-R-R-W-K-A-F-I-A-I-V-S-A-A-A-R-F-K-K-C. 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 \( K_a \) values for the calmodulin-peptide complexes by monitoring calmodulin-dependent changes in acrylodan fluorescence anisotropy. Mean values (10 determinations per data point) for acrylodan fluorescence anisotropy were estimated from enzyme kinetic data as described under "Experimental Procedures." For all three enzyme activities, \( K_a \) values determined from duplicate data sets were seen to vary by less than 10%. Duplicate determinations of \( K_a \) values for myosin light chain kinase and plant NAD kinase were within a factor of two; calcineurin \( K_a \) values are ± 50%.

*Calculated using a weighted nonlinear curve-fitting procedure.
^ 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 CaMA83–84 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 \( K_a \) 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 \( K_a \) 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, \( K_a \) values for binding of the peptide are increased 4–7-fold, and \( K_{cat} \) values for calcineurin are increased only 2–3-fold. All these differences in \( K_{act} \) or \( K_a \) 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 CaMA83, which contains only a single amino acid deletion, and either CaMA83–84 or CaMA81–84. Therefore, the major effect of the deletions on calmodulin function can be attributed to removal of a single glutamic acid from the cluster: E82–E83. 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 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).

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 bismaleimido-hexane 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
Deletions in the Calmodulin Central Helix

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 Ghu-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 calmodulin?

Stimulus-induced changes in intracellular Ca\textsuperscript{2+} ion are transient in nature, hence formation of Ca\textsuperscript{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 (Pereschni 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.

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