Identification of the Binding and Inhibition Sites in the Calmodulin Molecule for Ophiobolin A by Site-Directed Mutagenesis

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Ophiobolin A, a fungal toxin that affects maize and rice, has previously been shown to inhibit calmodulin by reacting with the lysine (Lys) residues in the calmodulin. In the present study we mutated Lys-75, Lys-77, and Lys-148 in the calmodulin molecule by site-directed mutagenesis, either by deleting them or by changing them to glutamine or arginine. We found that each of these three Lys residues could bind one molecule of ophiobolin A. Normally, only Lys-75 and Lys-148 bind ophiobolin A. Lys-77 seemed to be blocked by the binding of ophiobolin A to Lys-75. Lys-75 is the primary binding site and is responsible for all of the inhibition of ophiobolin A. When Lys-75 was removed, Lys-77 could then react with ophiobolin A to produce inhibition. Lys-148 was shown to be a binding site but not an inhibition site. The Lys-75 mutants were partially resistant to ophiobolin A. When both Lys-75 and Lys-77 or all three Lys residues were mutated, the resulting calmodulins were very resistant to ophiobolin A. Furthermore, Lys residues added in positions 86 and/or 143 (which are highly conserved in plant calmodulins) did not react with ophiobolin A. None of the mutations seemed to affect the properties of calmodulin. These results show that ophiobolin A reacts quite specifically with calmodulin.

Calmodulin is a small, acidic, Ca\(^{2+}\)-binding protein and is the major Ca\(^{2+}\) receptor in eukaryotic cells. Since its discovery as a phosphodiesterase protein activator (Cheung, 1971), many enzymes and proteins have been found to be capable of binding to and being regulated by calmodulin. The structures of Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free calmodulin have been resolved (Babu et al., 1988; Zhang et al., 1995). Comparison of these two structures has provided valuable information on how the binding of Ca\(^{2+}\) induces the exposure of hydrophobic surfaces in the N and C terminals of calmodulin. This conformational change allows Ca\(^{2+}\)-calmodulin to form a 1:1 complex with target proteins.

Ophiobolin A is a fungal metabolite and a phytotoxin produced by the plant pathogen Helminthosporium maydis Nisikado and Miyake and by other members of the same genus (Hesseltine et al., 1971). In roots of maize seedlings ophiobolin A causes a leakage of electrolytes and Glc from cells (Tipton et al., 1977). Ophiobolin A is believed to cause the symptoms of brown spot disease in rice (Narain and Biswal, 1992). In maize roots the cytotoxic effect of ophiobolin A was well correlated with calmodulin inhibition (Leung et al., 1985). When maize roots were treated with ophiobolin A, less active calmodulin was present in the root extract, indicating a possible in vivo inhibition of calmodulin by ophiobolin A. The cytotoxicity of ophiobolin A was attributed to its covalent binding to calmodulin. When calmodulin was incubated with ophiobolin A, the Tyr fluorescence of calmodulin was quenched (Leung et al., 1984). This quenching was correlated with the loss in calmodulin activity, and indicated a direct binding between calmodulin and the toxin. The e-amino group of Lys residues was implicated in the reaction with ophiobolin A (Leung et al., 1988), although the precise location of the reactive Lys in the calmodulin molecule is not known.

Using bovine-brain calmodulin as a model system, we report the identification of the Lys residues in the calmodulin molecule that react with ophiobolin A by site-directed mutagenesis. We found that Lys-75 and Lys-148 were the ophiobolin A-binding sites. However, only Lys-75 was responsible for the inhibitory effect of ophiobolin A. Removal of Lys-75 could confer resistance to the toxin. Lys-77 was another binding site, but was available only when Lys-75 was deleted or substituted by another amino acid. When Lys residues unique to plant calmodulins were introduced, ophiobolin A did not react with them.

MATERIALS AND METHODS

Ophiobolin A, snake venom 5’-nucleotidase, cAMP, malachite green, and most of the chemicals were from Sigma. Phenyl-Sepharose CL-4B, lysozyme, and DNase were from Pharmacia. Oligonucleotides were synthesized on a Gene Assembler (Pharmacia) and purified according to the recommended procedures of the manufacturer. Protein assay dye solution was from Bio-Rad. Bovine brains were obtained from local markets and transported to the laboratory on ice.

Protein Preparation

Calmodulin-deficient PDE from bovine brain was partially purified according to the method of Wallace et al.

Abbreviations: \(K_{\text{act}}\), the concentration of calmodulin required for half-maximal activation of PDE; PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase.
agar plates containing 100 JM105 cells. Transformants were selected on Luria-Bertani mixture was used to transform competent Escherichia coli Nco I and then ligated to pACA CTGAC/TCAA/GCTGACC/TGA-3'. 9 reaction the 5'-bovine-brain first-strand cDNA preparation. For the PCR calmodulin-coding region was isolated using PCR from a fragment of 450 bp that encompassed the entire gene. A cDNA was synthesized from total RNA from bovine brain by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The first strand of the cDNA was synthesized using a reverse-transcription system (Promega). The purified cDNA was digested with Bam HI, and then ligated to p9. Amplified cDNA was purified by agarose-gel electrophoresis. The purified cDNA was digested with Ncol and BamHI, and then ligated to pTrc99A vector (Pharmacia) previously digested with Ncol and BamHI. The ligation mixture was used to transform competent Escherichia coli JM105 cells. Transformants were selected on Luria-Bertani agar plates containing 100 µg/mL ampicillin and screened for clones that contained the calmodulin insert. The identities of the inserts were verified by DNA sequencing. One recombinant plasmid, designated pCam-Trc, contained the calmodulin cDNA sequence under the control of the trc promoter. The distance between the Shine-Dalgarno sequence and the translation-initiation codon ATG was found to be eight nucleotides. The predicted amino acid sequence was identical to the published bovine-brain calmodulin-amino acid sequence (Watterson et al., 1980). The bacterial clone that contained pCam-Trc was used for the expression of wild-type calmodulin.

Isolation of Bovine-Brain Calmodulin cDNA and Construction of a Calmodulin-Expression Plasmid

The following procedures produced a calmodulin-expression plasmid that directed the synthesis of calmodulin in bacteria. Total RNA was isolated from bovine brain by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The first strand of the cDNA was synthesized from total RNA from bovine brain using a reverse-transcription system (Promega). A cDNA fragment of 450 bp that encompassed the entire calmodulin-coding region was isolated using PCR from a bovine-brain first-strand cDNA preparation. For the PCR reaction the 5' and 3' primers were 5'-ACACCATGGCTGAC/TCAA/GCTGACC/TGA-3' and 5'-ACAGGATTCCATC/TTTC/TGCA/TGTATCAT-3', respectively. The boldface sequences are the Ncol and BamHI sites, respectively. Amplified cDNA was purified by agarose-gel electrophoresis. The purified cDNA was digested with Ncol and BamHI, and then ligated to pTrc99A vector (Pharmacia) previously digested with Ncol and BamHI. The ligation mixture was used to transform competent Escherichia coli JM105 cells. Transformants were selected on Luria-Bertani agar plates containing 100 µg/mL ampicillin and screened for clones that contained the calmodulin insert. The identities of the inserts were verified by DNA sequencing. One recombinant plasmid, designated pCam-Trc, contained the calmodulin cDNA sequence under the control of the trc promoter. The distance between the Shine-Dalgarno sequence and the translation-initiation codon ATG was found to be eight nucleotides. The predicted amino acid sequence was identical to the published bovine-brain calmodulin-amino acid sequence (Watterson et al., 1980). The bacterial clone that contained pCam-Trc was used for the expression of wild-type calmodulin.

Site-Directed Mutagenesis

Site-directed mutations of individual Lys residues in calmodulin were performed by the inverse PCR method of Hemsley et al. (1989). A pair of oligonucleotide primers complementary to the calmodulin sequence at the site of mutagenesis were designed so that they would line up in a back-to-back fashion on opposite strands of the template. One of the primers carried the desired mutation. The uncut calmodulin-expression plasmid was used as the template in the PCR. Amplified linear DNA was self-ligated to regenerate a circular plasmid with the mutation incorporated. Three series of mutations were created in the Lys-75, Lys-77, and Lys-148 of the calmodulin molecule. They were the single-amino acid mutants (K75Δ, K75Q, K75R, K77Δ, K77A, K77Q, and K77R), the double-amino acid mutants (K75Δ, K75Δ,148R, and K77Δ,148R), and the triple-amino acid mutants (K75,77Δ,148R, K75,77,148A, K75,77,148Q, and K75,77,148R), where Δ stands for deletion. For the single-amino acid mutants, K75Δ indicates that Lys-75 was deleted, K75Q indicates that Lys-75 was changed to Gln, and K75R indicates that Lys-75 was changed to Arg. The same meaning is applied to Lys-77 and Lys-148 mutants. For the double-amino acid mutants, K75,77Δ indicates that both Lys-75 and Lys-77 were deleted, K75Δ,148R indicates that Lys-75 was deleted and Lys-148 was changed to Arg, and K77Δ,148R indicates that Lys-77 was deleted and Lys-148 was changed to Arg. For the triple-amino acid mutants, K75,77Δ,148R indicates that Lys-75 and Lys-77 were deleted and Lys-148 was changed to Arg; K75,77,148Δ indicates that Lys-75, Lys-77, and Lys-148 were deleted; K75,77,148Q indicates that Lys-75, Lys-77, and Lys-148 were changed to Gln; and K75,77,148R indicates that Lys-75, Lys-77, and Lys-148 were changed to Arg. The deletion mutation was generated by amplification with a pair of oligonucleotide primers that spanned both sides of the Lys codon (AAA). Therefore, the Lys codon was not included in the amplified DNA product. For the Gln and Arg substitutions, the AAA codon was replaced by CAG and CGT in the oligonucleotides, respectively. A silent mutation was introduced into the K75 mutants to create a unique MscI site for screening purposes. This silent mutation was not introduced in subsequent mutagenesis. To obtain the double-amino acid mutants K75,77Δ and K75Δ,148R, the single-amino acid mutant K75Δ was used as the PCR template. For K77Δ,148R, the K77Δ was the PCR template. Likewise, the triple-amino acid mutants were obtained using single- or double-amino acid mutants as PCR templates. Using the triple-amino acid mutant K75,77,148R as the PCR template, additional Lys residues were introduced at positions 86 and 143 of the calmodulin molecule as single-addition (R86K and Q143K) and double-addition (R86,Q143K) mutants. Thus, mutant R86K has Arg-86 changed to Lys, mutant Q143K has Gln-143 changed to Lys, and mutant R86,Q143K has Arg-86 and Gln-143 changed to Lys, in addition to the three mutations at Lys-75, Lys-77, and Lys-148. The CGC codon (for Arg) at position 86 and the CAG codon (for Gln) at position 143 were replaced by the AAA codon (for Lys) in the mutagenesis primers. The PCR took place in a 100-µL reaction mixture containing 20 mM Tris-HCl, pH 8.8, at 25°C, 10 mM KCl, 10 mM (NH4)2SO4, 3 mM MgSO4, 0.1% Triton X-100, 100 µg/mL.
BSA, 400 μM of each deoxyribonucleotide triphosphate, 5 ng of the template plasmid, 1 μM of each oligonucleotide primer, and 1 unit of DNA polymerase (Vent, New England Biolabs). This reaction mixture was incubated for 25 cycles (1 cycle = 94°C for 1 min, 60°C for 1 min, and then 72°C for 5 min) followed by a 10-min incubation at 72°C. The products of the reaction were purified by agarose-gel electrophoresis. A portion of the agarose-gel-purified PCR product was phosphorylated at the 5' ends and self-ligated in 20 μL of 70 mM Tris-HCl, pH 7.6, at 25°C, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP, 5 units of T4 polynucleotide kinase (New England Biolabs), and 200 units of T4 DNA ligase (New England Biolabs) at 15°C for 16 h. The ligation mixture was used to transform competent E. coli JM105 cells. Transformants were selected by plating on Luria-Bertani agar plates containing 100 μM ampicillin, and 200 units of T4 DNA ligase (New England Biolabs) at 15°C for 16 h. The ligation mixture was used to transform competent E. coli JM105 cells. Transformants were selected by plating on Luria-Bertani agar plates containing 100 μg/mL ampicillin and then screened for the expression of calmodulin. The presence of the desired mutations in the plasmid were confirmed by sequencing the entire coding sequence. For each mutagenesis, more than 80% of the clones selected by ampicillin resistance contained the desired mutation.

Expression and Purification of Wild-Type and Mutated Calmodulins

Bacterial expression and purification of wild-type and mutated calmodulins were performed using the method of Putkey et al. (1985) with modifications. A single colony of E. coli JM105 carrying the appropriate expression plasmid was used to inoculate 50 mL of Luria-Bertani medium containing 100 μg/mL ampicillin. After overnight growth at 37°C, 20 mL was used to inoculate 1 L of 2× YT medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, pH 7.0) containing 100 μg/mL ampicillin. The culture was incubated at 37°C for 2 h with shaking. Expression of calmodulin was induced by the addition of isopropylthio-β-galactoside to a concentration of 0.1 mM. The culture was incubated for another 6 h. The cells were harvested by centrifugation (4000g, 5 min, 4°C). The cell pellet was washed twice in 200 mL of 50 mM Tris-HCl, pH 7.5, and then resuspended in 100 mL of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. Lysozyme was added to 200 μg/mL, and the suspension was incubated at 4°C for 30 min. MgCl₂ and DNase were added to concentrations of 10 mM and 10 μg/mL, respectively. The mixture was incubated at 4°C for another 2 h. CaCl₂ was added to a concentration of 10 mM.

The lysate was then placed in a boiling-water bath for 5 min and then on ice for 10 min. The suspension was cleared by centrifugation at 31,000g for 30 min at 4°C. The supernatant was applied to a 5-mL phenyl-Sepharose CL-4B column equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 1 mM DTT, and 0.5 mM PMSF. The column was then washed with 50 mL of the same buffer and then with 50 mL of the buffer containing 0.5 mM NaCl. Calmodulin was eluted from the column with 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. Eluted protein was dialyzed extensively against deionized water, concentrated, and stored at −20°C. The yields were 10 to 16 mg of calmodulin per liter of cell culture, which is comparable to the yields of other protocols (Putkey et al., 1985; Roberts et al., 1985; Rhyner et al., 1992).

N-terminal sequencing of the recombinant wild-type calmodulin by a protein-sequencing system (model G1005A, Hewlett-Packard) revealed that the recombinant wild-type calmodulin has a nonacetylated Ala as the first residue (data not shown). This is the same first amino acid as in natural bovine-brain calmodulin, although the Ala in the latter is acetylated. Therefore, the same numbering system was used for recombinant calmodulin in this study.

Binding Measurements

Binding between ophiobolin A and calmodulin was monitored by the increase in A272. It has been shown that a new chromophore at 272 nm is formed quantitatively when ophiobolin A reacts with calmodulin (Leung et al., 1988). The binding experiments were carried out in 5-mL glass culture tubes (Fisher Scientific). In these experiments the calmodulin was in 0.15 mL of 40 mM Tris-HCl, pH 7.5, and 1 mM CaCl₂. Various concentrations of ophiobolin A were added as 1 or 5 mM solutions in methanol using a syringe (Hamilton Co., Reno, NV). After incubation at room temperature for 2 h, the A272 was measured by a UV-visible spectrophotometer (Biochrom 4060, Pharmacia LKB). The absorbance was corrected for the absorbance of the calmodulin and that of ophiobolin A to obtain the absorbance of the calmodulin-ophiobolin A complex. The extent of ophiobolin A binding to calmodulin was calculated using the molar extinction (19,200 M⁻¹ cm⁻¹ at 272 nm) of the conjugated enamine product (Leung et al., 1988).

Recombinant DNA Methods

DNA fragments were purified by agarose gel electrophoresis and eluted using a kit (Sephaglas BandPrep, Pharmacia). Nucleotide sequencing was performed by the dideoxy chain-termination method (T7Sequencing kit, Pharmacia). Plasmid DNA was isolated by the alkaline lysate method, as described by Sambrook et al. (1989).

RESULTS

Comparison of Bovine-Brain and Recombinant Wild-Type Calmodulin

The recombinant wild-type calmodulin we prepared is identical in many ways to the natural bovine-brain calmodulin: it can be purified by phenyl-Sepharose column chromatography; it has a similar UV spectrum; and it can activate PDE and be inhibited by ophiobolin A to the same extent. The concentration of ophiobolin A required for half-maximal inhibition for the recombinant wild-type calmodulin (Fig. 1, middle) was found to be 1.5 μM.

Effects of Single-Amino Acid Mutations

In a previous study it was suggested that ophiobolin A may interact with Lys residues 75 and 148 in the calmodulin molecule (Leung et al., 1988). Therefore, we started by...
introducing mutations at these two positions in the calmodulin molecule. To assess the effects of mutations on calmodulin, three parameters were used: (a) the ability to activate PDE, (b) the extent of inhibition by ophiobolin A, and (c) the extent of binding by ophiobolin A. Figure 1 (top) shows the effect of these mutations in the activation of PDE. Deletion of Lys-75 (K75Δ) or substitution by Gln (K75Q) or Arg (K75R) had little effect on the activation of PDE. These mutant calmodulins could maximally activate PDE with $K_{\text{act}}$ values of 5, 8.5, and 7 nM, respectively. The mutants K148Δ, K148Q, and K148R could also activate PDE to a maximum with $K_{\text{act}}$ values of 8.5, 6.5, and 6.5 nM, respectively. The PDE prepared in this study could be activated approximately 6.5-fold by natural bovine-brain calmodulin with a $K_{\text{act}}$ of 6.5 nM.

The mutants were then assayed for their extent of inhibition by ophiobolin A. As shown in Figure 1 (middle), the K75 mutants (K75Δ, K75Q, and K75R) were only partially inhibited by the toxin. The concentration of ophiobolin A required for half-maximal inhibition increased from 1.5 μM for wild-type calmodulin to 9 μM for the mutants. In contrast, the inhibition curves of the K148 mutants (K148Δ, K148Q, and K148R) were almost identical to that of the wild-type calmodulin. Therefore, Lys-75 is a site for ophiobolin A inhibition and Lys-148 is not.

The mutants were further analyzed for their ophiobolin A-binding capacity. The results show that the wild-type calmodulin bound 2 mol of ophiobolin A, the K75 mutants bound about 1.7 mol, and the K148 mutants bound about 1.4 mol of ophiobolin A per mol of calmodulin (Fig. 1, bottom). The decrease in binding in the K75 mutants was small. The decrease in the K148 mutants was large and indicates that Lys-148 is a binding site for ophiobolin A.

If Lys-75 were the only site of inhibition by ophiobolin A, then removing it by deletion or substitution should have removed all of the inhibition. However, there was still

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Figure 1. The effects of single-amino acid mutations on the ability to activate PDE (top), the inhibition by ophiobolin A (middle), and the extent of ophiobolin A binding of calmodulin (bottom). Top, PDE activation assay was done in a final reaction volume of 90 μL containing 0.0015 unit bovine brain PDE, 0.03 unit 5′-nucleotidase, 40 mM Tris-HCl, 40 mM imidazole, 5 mM magnesium acetate, and 0.5 mM CaCl$_2$, pH 7.5. Wild-type or mutant calmodulins were added to the concentrations indicated. The reaction mixture was preincubated at 30°C for 1 min before the addition of cAMP to a final concentration of 1.2 μM to start the enzyme reaction. After 30 min at 30°C, the reaction was stopped by the addition of 910 μL of water and the amount of phosphate was measured. Maximal activation is the PDE activity at 45 nM wild-type calmodulin minus the basal activity (activity in the absence of calmodulin). The extent of phosphodiesterase activation by lower concentrations of wild-type or mutant calmodulins was expressed as a percentage of the maximal activation. The measurements were separated into two experiments. Middle, Inhibition of calmodulins by ophiobolin A was measured by the decrease in the activation of PDE. The reaction conditions were as in the top graph, except that the calmodulin concentration was kept at 15 nM. Ophiobolin A was added as a 1 or 5 mM solution in methanol to the desired concentrations. The reaction mixtures were then incubated at 30°C for 30 min before the addition of cAMP. The PDE activity minus the basal activity in the absence of ophiobolin A was taken as the maximal activity. PDE activity at each concentration of ophiobolin A was expressed as a percentage of the maximal activity. All measurements were made in two experiments. Bottom, Extent of ophiobolin A binding to each mole of calmodulin. Aliquots of 10 μM calmodulin in 40 mM Tris-HCl, pH 7.5, and 1 mM CaCl$_2$ were incubated with various concentrations of ophiobolin A. All measurements were made in two experiments. All experiments were repeated at least two times. Wild-type calmodulin; □, K75Δ; X, K75Q; ■, K75R; □, K77Δ; △, K148Δ; Δ, K148Q; ▼, K148R.
some inhibition left in the K75 mutants (Fig. 1, middle),
suggesting that an inhibition site other than Lys-75 and 
Lys-148 may be responsible for the inhibition left in the K75 
mutants. Lys-77, which is in proximity to Lys-75, could be 
a potential site of inhibition, because in the three-
dimensional structure of calmodulin, Lys-75 and Lys-77 
were shown to be in a similar environment (Babu et al., 
1988). Therefore, a Lys-77 deletion mutant (K77Δ) was 
prepared. This mutant calmodulin could activate PDE nor-
mally (Fig. 1, top) and bound about 1.7 mol of ophiobolin 
A per mol of calmodulin, like the K75 mutants (Fig. 1, 
bottom). The mutant could be inhibited by ophiobolin A, 
like the wild-type calmodulin (Fig. 1, middle). This result 
shows that Lys-77 is not a site of inhibition.

Effects of Double-Amino Acid Mutations

Next, we studied the effect of changing two Lys residues 
at a time. Three mutants (K75,77Δ; K77Δ,148R; and 
K75Δ,148R) were made. They could activate PDE to near 
maximum in spite of the mutations and their $K_{act}$ values 
were 4, 4, and 5.5 nM, respectively (Fig. 2, top). The 
ophiobolin A inhibition assay showed that mutant K75,77Δ 
was only minimally inhibited by ophiobolin A (Fig. 2, 
middle). This resistance was most prominent at lower con-
centrations of ophiobolin A. At 20 μM ophiobolin A the 
mutant calmodulin was inhibited by less than 10%, 
whereas the wild-type calmodulin was inhibited by 90%. 
Mutants K77Δ,148R and K75Δ,148R were produced to dis-
tinguish the effect of these two residues without the con-
tribution of Lys-148. It was found that K77Δ,148R was 
inhibited by ophiobolin A as easily as the wild-type cal-
modulin (Fig. 2, middle). Like the K75 single-amino acid 
mutants, K77Δ,148R exhibited partial resistance to the 
toxin, but the extent of resistance was larger in K75Δ,148R 
(Fig. 2, middle).

When the ophiobolin A-binding profiles of these double-
amino acid mutants were determined, it was found that 
these three mutants bound only 1 mol of ophiobolin A per 
mol of calmodulin (Fig. 2, bottom).

Effects of Triple-Amino Acid Mutations

To explore the combined effect of Lys-75, Lys-77, and 
Lys-148 in the ophiobolin A inhibition, triple-amino acid 
mutants (K75,77Δ,148R; K75,77,148Δ; K75,77,148Q; and 
K75,77,148R) were produced. In these mutants the three 
Lys residues were removed either by deletion or substitu-
tion. All of the mutants could activate PDE to approxi-
mately maximum. The $K_{act}$ values were 4.5, 5.5, 6.5, and 7 
nM, respectively (Fig. 3, top). These mutants exhibited a 
great deal of resistance to ophiobolin A inhibition (Fig. 3, 
middle). For unknown reasons, mutants with deletions in 
Lys-75 and Lys-77 provided more resistance than mutants 
with substitutions in these two positions. In the binding 
experiment, the extent of ophiobolin A binding to these 
triple-amino acid mutants was about 0.6 mol of ophiobolin 
A per mol of calmodulin (Fig. 3, bottom). This fractional 
binding was probably caused by the binding of ophiobolin 
A to some nonspecific sites, because the initial rate of

![Figure 2. Effects of double-amino acid mutations on the ability to activate PDE (top), the inhibition by ophiobolin A (middle), and the extent of ophiobolin A binding of calmodulin (bottom). Experimental details are as described in the legend to Figure 1. All measurements were made in one experiment. All experiments were repeated at least two times. ●, Wild-type calmodulin; ○, K75,77Δ; ×, K75Δ,148R; ■, K77Δ,148R.](image-url)
binding was very slow. For example, at 100 μM ophiobolin A, the rate of binding of ophiobolin A to the triple-amino acid mutant K75,77Δ,148R was at least 30 times smaller than the binding rate to the wild-type calmodulin (data not shown).

Effects of Introducing Additional Lys Residues

The results presented above established that Lys-75 in the calmodulin molecule was responsible for the inhibitory action of ophiobolin A. To determine if other Lys residues in plant calmodulin also react with ophiobolin A, additional Lys residues were introduced in positions 86 and/or 143 of the triple-amino acid mutant K75,77,148R. These two Lys residues are unique in plant calmodulins and are present in nearly all plant calmodulins characterized so far (Poovaiah and Reddy, 1993). As shown in Figure 4, the mutant calmodulins (R86K, Q143K and R86, Q143K) were nearly identical to the parental molecule (K75,77,148R) in terms of PDE-activating ability, the extent of inhibition by ophiobolin A, and the extent of binding by ophiobolin A. This indicates that the additional Lys residues in the mutant calmodulins do not react with ophiobolin A.

DISCUSSION

We used site-directed mutagenesis to locate the ophiobolin A-binding sites in the calmodulin molecule. This approach is based on the idea that the removal of the reactive Lys residues should result in a decrease in the extent of inhibition and the extent of binding by ophiobolin A in the calmodulin molecule. Three types of mutations were made: deletion of the Lys residue, changing Lys to Gln, and changing Lys to Arg. Gln and Arg were chosen because both contain nitrogen in the side chain, and this nitrogen does not form a Schiff base with carbonyl groups like it does in Lys. Therefore, the reaction between calmodulin and ophiobolin A was eliminated because the reaction between calmodulin and ophiobolin A was proposed to be a Schiff-base reaction (Leung et al., 1988).

Gln has an α-helix-forming propensity similar to that of Lys (Maxfield and Scheraga, 1979) and would preserve the structure at the Lys site. With Arg, the positive charge in the side chain is preserved. All mutant calmodulins show properties in common with those of the natural bovine-brain calmodulin. They could be purified by Ca²⁺-dependent phenyl-Sepharose chromatography, indicating that the mutations had not affected the Ca²⁺-induced exposure of hydrophobic domains in the calmodulin molecule. The UV spectra of all mutant calmodulins were similar to that of the natural bovine-brain calmodulin. We also looked at the electrophoretic mobility of the mutant calmodulins under denaturing and nondenaturing conditions, and the results did not suggest any great change in the structure of the mutant calmodulins (data not shown).

Using this series of mutants we confirmed the previous suggestion that Lys-75 and Lys-148 are the binding sites for ophiobolin A (Leung et al., 1988), and found that Lys-77 is also a binding site. Binding means the presence of a covalent interaction between ophiobolin A and the e-amino
group of the Lys residue (Leung et al., 1988). The double-
amino acid mutants K75,77Δ; K77Δ,148R; and K75Δ,148R
(Fig. 2, bottom) bind 1 mol of ophiobolin A per mol of
calmodulin over a wide range of ophiobolin A concentra-
tions, showing that each of the three binding sites can bind
one molecule of ophiobolin A and has similar affinity for
the toxin.

For K75,77Δ, ophiobolin A was bound to Lys-148; for
K75Δ,148R, it was bound to Lys-77; and for K77Δ,148R, it
was bound to Lys-75. These assignments are supported by
the results with the triple-amino acid mutants. When all
three Lys residues were removed, the binding was almost
abolished (Fig. 3, bottom). The residual fractional binding
in the triple-amino acid mutants was probably caused by
nonspecific binding, because the rates of these fractional
binding reactions were much slower than that of the spe-
cific binding reactions with the wild-type calmodulin.
These nonspecific binding sites are presumably the other
Lys residues in the calmodulin molecule.

Although there are three specific binding sites for
ophiobolin A, the data show that only two sites are used,
because the maximum number of moles of ophiobolin A
bound to each mole of wild-type calmodulin was 2. The
same molar ratio was obtained for the natural bovine-brain
calmodulin (Leung et al., 1988). A possible explanation is
that one of the three sites is hindered. This conclusion is
based on the fact that the maximum binding was only
about 1 mol of ophiobolin A per mol of calmodulin for the
K148 single-amino acid mutants (Fig. 1, bottom), in which
Lys-148 was removed and Lys-75 and Lys-77 remained
intact in the calmodulin molecule. This binding ratio sug-
jects that although Lys-75 and Lys-77 can bind one mole-
cule of ophiobolin A independently, they do not combine
to give two binding sites for ophiobolin A. Lys-77 is the
most probable hindered site. Lys-75 is not the hindered
site; the inhibition assay revealed it to be the main binding
site. It is possible that the binding of ophiobolin A to Lys-75
sterically prevents another molecule of ophiobolin A from
binding to Lys-77 because Lys-75 and Lys-77 are very close
to each other.

The fractional binding in the single mutants (Fig. 1,
bottom) might be explained as follows. The 1.4 value for
the K148 mutants might have been caused by nonspecific
binding added to the specific binding value of 1.0. The 1.7
value for K75 and K77 mutants might have been attribut-
able to some structural changes that lowered the binding
capacity of the mutants from a value of 2.0.

The binding of ophiobolin A to calmodulin does not
necessarily inhibit calmodulin. Inhibition means the loss of
the ability to activate PDE. For example, Lys-148 can bind
ophiobolin A but does not seem to be an inhibition site,
because the removal of Lys-148 in each of the K148 single-
amino acid mutants did not reduce the inhibition by
ophiobolin A.

The results show that Lys-75 alone was responsible for
all of the inhibition by ophiobolin A. Whenever Lys-75 was
removed, either by deletion or by substitution, the inhibi-
tion by ophiobolin A was reduced. Also, whenever Lys-75
was present in the calmodulin molecule, whether Lys-77

Figure 4. Effects of introducing additional Lys residues on the ability
to activate PDE (top), the inhibition by ophiobolin A (middle), and
the extent of ophiobolin A binding of calmodulin (bottom). Experi-
mental details are as described in the legend to Figure 1. All exper-
iments were repeated once. ●, Wild-type calmodulin; ○, K75,77,148R; ×, R86K; ■, Q143K; □, R86,Q143K.
and Lys-148 were present, the calmodulin was as easily inhibited by ophiobolin A as wild-type calmodulin.

Although Lys-77 can bind ophiobolin A, it does not seem to be a site of inhibition. If Lys-77 were another site of inhibition, there should have been a reduction of inhibition whenever Lys-77 was removed from the calmodulin. No such reduction in inhibition was observed in any of the K77 mutants when Lys-75 was intact. The mutants K77Δ and K77Δ,148R (Figs. 1 and 2, middle panels) were as easily inhibited by ophiobolin A as the wild-type calmodulin. This result is consistent with the finding that Dictyostelium discoideum calmodulin, which contains a Glu at position 77 and a Lys at position 75, was as easily inhibited by ophiobolin A as the bovine-brain calmodulin (Leung et al., 1988). However, when Lys-75 is removed, Lys-77 seems to become a site of inhibition. This is suggested by the partial inhibition left in the K75 single-amino acid mutants (Fig. 1, middle), which was caused by the binding of ophiobolin A to Lys-77, because when Lys-77 was also removed, as in the double-amino acid mutant K75,77Δ, the partial inhibition was also removed and the mutant became very resistant to ophiobolin A.

Calmodulins from many sources contain seven Lys residues and one trimethyl-Lys residue. Of the seven Lys residues, Lys-75 and Lys-148 have been shown to react with a number of chemical reagents (Jackson and Puett, 1984; Faust et al., 1987; Newton and Klee, 1989). The interaction of ophiobolin A with calmodulin is in many respects similar to that of synthetic, therapeutic phenothiazines. Both compounds bind calmodulin in a \( \text{Ca}^{2+} \)-dependent manner and in a 2:1 molar ratio. The phenothiazines norchlorpromazine isoiodioyanate (Newton et al., 1983) and 10-(3-propionyloxy succinimide)-2-(trifluoromethyl) phenothiazine (Faust et al., 1987) were also reported to label Lys-75 and Lys-148. It is interesting that a natural product possesses properties similar to those of a synthetic compound. However, ophiobolin A represents a calmodulin inhibitor of a different chemical class (a sesterterpenoid). It can be a prototype for further synthesis of a highly specific calmodulin inhibitor.

Although the current research was done using calmodulin from bovine brain, the results can most likely be extended to plant calmodulins. Ophiobolin A has been shown to inhibit spinach and maize calmodulin (Leung et al., 1984, 1985) in the same manner as bovine-brain calmodulin. The sequence and structure of calmodulin is conserved. Maize, rice, and spinach calmodulins also contain Lys in positions equivalent to Lys-75, Lys-77, and Lys-148 of bovine-brain calmodulin (Poovaiah and Reddy, 1993). More importantly, the introduction of Lys residues unique to plant calmodulins does not increase the inhibition and binding by ophiobolin A (Fig. 4). The results presented in this paper show that ophiobolin A reacts quite specifically with calmodulin and that this could be the mechanism of action of ophiobolin A.

Although we have suggested that the introduced mutations did not cause any large structural changes in the mutant calmodulins, small changes in the local environment of the molecule most likely occurred. These changes may have caused some slight variations in the interaction of the mutant calmodulins with the PDE enzyme or ophiobolin A. If this is true, then some of the variations between mutants may be explained by these local molecular variations. For example, the unexpected slightly higher resistance to ophiobolin A in K75Δ,148R than in K75Δ (Figs. 1 and 2, middle panels) might have been caused by a slight variation in the molecular environment between Arg and Lys at residue 148. The same argument may be applied to explain why the triple mutants with Lys-75 and Lys-77 deleted were more resistant to ophiobolin A than those with substitutions in these two positions (Fig. 3, middle).

We have not explored in detail the structural changes that may have occurred as a result of mutagenesis and the role of hydrophobic amino acid residues in the interaction between ophiobolin A and calmodulin. Further studies of these two aspects will give a more complete picture of the interaction between these two interesting molecules.

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