Calmodulin-binding Proteins Also Have a Calmodulin-like Binding Site within Their Structure

THE FLIP-FLOP MODEL*

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The flip-flop model is a mechanistic model proposed to describe how calmodulin activates enzymes. One prediction based upon this model is that calmodulin-activated enzymes would contain a calmodulin-like binding site which, among other attributes, would bind the peptide melittin. Five purified calmodulin-activated enzymes, namely calcineurin, myosin light chain kinase, phospholipase b kinase, phosphodiesterase, and NAD kinase, were all found to bind biotinylated melittin and to also bind an antimelittin antibody and biotinylated calmodulins. Using gel blots of crude tissue extracts (rat brain and Arabidopsis), most proteins did not bind any of the probes and thus do not have these characteristics. However, among those which bind any of these probes, a strong correlaton was found between those proteins which bind biotinylated calmodulins and those which bind melittin and antimelittin. Gel blots of phosphorylase b kinase demonstrate that the α, β, and γ subunits all bind calmodulin and melittin. A putative calmodulin-like binding site sequence was identified in eight enzymes or subunits which may play an important role in both melittin binding and calmodulin-dependent regulation of these enzymes.

Calmodulin is a Ca2+-dependent activator of numerous enzymes which are critical to cellular metabolism (1, 2). Many of these enzymes have also been shown to be activated by limited proteolysis with trypsin or by acidic lipids such as oleic acid (2). In either of these cases, enzyme activity is Ca2+-independent. Calmodulin also binds cationic phenothiazine drugs such as trifluoperazine (3, 4) and certain basic peptides such as the bee venom peptide, melittin (5), in a Ca2+-dependent manner, and binding of these agents antagonizes enzyme activation. In this report, we will show that calmodulin-binding proteins also bind melittin. This behavior is predicted by a mechanistic model we have developed.

As the model in Fig. 1 shows, calmodulin binds Ca2+ and undergoes a conformational change to a conformer capable of binding antagonists or enzymes. Such a conformational change has been documented using various physical methods (2). In this conformation, calmodulin is thought to have several binding sites which have the properties of being hydrophobic and anionic, and these sites are thought to play a major role in the binding of antagonists and enzymes. That anionic lipids can activate (i.e. are agonists of) calmodulin-activated enzymes is thought to be due to these hydrophobic anions mimicking the hydrophobic, anionic sites on calmodulin. This also explains why hydrophobic cations such as trifluoperazine bind to Ca2+-calmodulin and antagonize enzyme activation. There appear to be at least four trifluoperazine-binding sites on calmodulin (4) although various studies with a variety of phenothiazines have reported five to eight (6, 7) or even 26 (3) phenothiazine-binding sites. These sites, or some fraction of them, are thought to also be involved in the binding of antagonist peptides such as melittin and to the calmodulin-binding regions of enzymes. Enzyme-derived, putative calmodulin-binding peptides have been identified for myosin light chain kinases (8–10), phosphofructokinase (11, 12), phosphorylase b kinase (11, 13–15), Ca2+-ATPase (16), and calmodulin kinase II (17). These peptides all possess basic amino acids adjacent to hydrophobic ones and this cationic, amphipathic motif is also shared by melittin and other calmodulin antagonist peptides. The trifluoperazine-binding sites participate in antagonist peptide binding since a single melittin binds to calmodulin (5) and this binding displaces two of the four equivalents of trifluoperazine which bind to calmodulin (4); also, peptide, drug, and enzyme binding to calmodulin all protect Lys39 and Lys44 from acetylation (18–20). Since trifluoperazine is also an antagonist of calmodulin activation, it is thought to compete with the enzymes for the hydrophobic, anionic sites on calmodulin.

Calmodulin-activated enzymes are envisioned (Fig. 1) to be composed of a regulatory domain containing the calmodulin-binding site and a catalytic domain. In the absence of calmodulin, the regulatory domain inhibits catalysis and thus these enzymes are "internally inhibited." Calmodulin binds to the regulatory domain and this binding removes the internal inhibition. Activation by limited proteolysis may be explained by a trypsin-labile peptide bond between the regulatory and catalytic domains, and thus proteolysis can also remove the internal inhibition. Several predictions can be based on this model. 1) Calmodulin-activated enzymes should all possess cationic, amphipathic regions which are similar in these characteristics to melittin and the other calmodulin antagonist peptides. We will call this region the calmodulin-binding site of CBS1. The presence of such a site is supported by several different studies. The enzyme-derived, putative calmodulin-binding peptides described above certainly agree with this model.

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1 The abbreviations used are: CBS, calmodulin-binding site; CLBS, calmodulin-like binding site; BC1, biotinylated calmodulin prepared by the method in Ref. 27; BM, biotinylated melittin; AM, anti-melittin antibody; MLCK, myosin light chain kinase; CN, calcineurin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin.
prediction. Other studies show that anti-melittin, an antibody raised against melittin, binds to calmodulin-binding proteins (21). While the antibody binding is weak in at least some cases, this suggests that there is enough general similarity between melittin and the calmodulin-binding region of enzymes that antibody cross-reactivity occurs. 2) Calmodulin-activated enzymes are predicted to also contain at least one site which binds cationic, amphipathic regions of peptide sequence. Since the binding of such sequences is also a property of calmodulin (e.g. calmodulin binds melittin), we call this the calmodulin-like binding site or CLBS. While other variations of the model can be constructed, the model in Fig. 1 predicts the (hydrophobic, cationic) CBS also has a (hydrophobic, anionic) binding site within the enzymes structure, the CLBS. Evidence for this last prediction is almost totally lacking, and it is this prediction that the experiments presented here were designed to test.

Here we confirm that calmodulin-activated enzymes do contain a melittin-like region recognized by anti-melittin and that furthermore, they possess a binding site for cationic amphipathic peptides such as melittin. Furthermore, we show that this is also true for an enzyme like NAD kinase which is neither activated by limited proteolysis or oleic acid (22) and thus has some attributes which appear contrary to the model.

**MATERIALS AND METHODS**

**Enzymes**—Myosin kinase from rabbit skeletal muscle was the generous gift of Dr. James T. Stull (Department of Physiology, University of Texas Health Sciences Center, Dallas, TX). Calceinurin (phosphoprotein phosphatase 2b) was the generous gift of Dr. Wai Y. Cheung and rabbit muscle phosphorylase b kinase was the generous gift of Dr. Gerald M. Carlson, both of our department. NAD kinase was purified from pea seedlings as previously described (23). Phosphodiesterase was purified from bovine brain by the procedure of Sharma et al. (24). One unit of activity was defined as that which hydrolyzes $\lambda$ of $3',5'$-cAMP/min at $30^\circ$C and pH 7.0.

**Preparation of Biotinylated Probes**—Calmodulin was purified from porcine brain as previously described (25). Calmodulin was biotinylated with the Bio-sys reagent and stored frozen in 20% glycerol.

**Melittin-Sepharose**—The calmodulin-Sepharose as described (21) and diluted to twice the volume of the serum used initially. The diluted antibody was then dialyzed (21), aliquoted, and stored frozen. Antibody concentrations are reported in terms of dilutions relative to the original serum volumes.

**Peptide Maps**—30 nmol each of calmodulin, BC1, or BC2 were lyophilized and dissolved in 50 mM NH$_4$HCO$_3$, 1 mM 2-mercaptoethanol, and desalted on a Sephadex G-25 column equilibrated in this last solvent. Concentration was determined by absorption, and 0.2-mg aliquots were lyophillized and stored at $-20^\circ$C. The extent of biotinylation of the melittin could not be directly investigated but was indirectly investigator using peptide mapping (see below).

**Anti-melittin Antibody**—Rabbit serum containing the anti-melittin antibody was the generous gift of Marcia Kaetzel and John R. Dedman (Department of Physiology and Cell Biology, University of Texas Health Sciences Center, Houston, TX). The anti-melittin antibody was biotinylated with N-hydroxysuccinimidyl-biotin at pH 6 and 2 mM CaCl$_2$ by the procedure of Mann et al. (26). We refer to this derivative as BC1. One unit of activity was defined as that which hydrolyzes $\lambda$ of $3',5'$-cAMP/min at $30^\circ$C, 1 mM EDTA, and pH 8.0. The homogenate was then centrifuged at 100,000 x g for 30 min, and the supernatant was filtered through a 0.45-µm pore Millipore filter. The extracts were dialyzed versus 50 mM NH$_4$HCO$_3$, 1 mM 2-mercaptoethanol, and then washed with the protein concentration was determined (28) using bovine serum albumin as the standard. Aliquots containing 1 mg of protein were lyophillized and stored frozen. Immediately prior to use, the lyophillized extracts were dissolved in sample buffer and immersed in boiling water for 2 min. The boiled samples were applied to 12% sodium dodecyl sulfate-polyacrylamide gels (29) and electrophoresed. The gels were then removed and the proteins blotted over to nitrocellulose using essentially the procedure of Towbin et al. (30) as described in the Bio-Rad TransBlot instruction manual. Gel blotting was for 1 h at 100 V using 25 mM Tris, 192 mM glycine, 20% methanol. The nitrocellulose was then either stained for protein with 0.1% Amido Black, 45% methanol, 10% acetic acid or was incubated overnight at 4°C in 1 mg/ml bovine serum albumin in TTBS for subsequent experiments. The blocked gel blot was washed once in 10 ml of BSA/Ca/TTBS and then incubated for 1 h with either 0.25 µg BC1, 0.27 µg BC2, 0.15 µg BM, or a 1:20 dilution of the anti-melittin antibody. The dilutions were prepared in BSA/Ca/TTBS. The blot was then washed three times with 10 ml of BSA/Ca/TTBS and incubated with avidin or goat anti-rabbit immunoglobulin G conjugates with alkaline phosphatase or horseradish peroxidase as appropriate and specified in the figures and stained for activity.

**FIG. 1. The flip-flop model.** CaM, calmodulin.
dialyzed versus 50 mM NH₄HCO₃, 0.1 mM EGTA. These samples and 30 nmol of BM were then diluted to 6 ml with this last buffer, and 0.06 ml of 50 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Worthington Biochemicals) in 1 mM HCl was added. Digestion was at room temperature overnight. The samples were then lyophilized and dissolved in 0.5 ml of 0.1% trifluoroacetic acid (buffer A). 50 μl of the samples was injected onto an Econosphere C4 column (7-μm bead diameter, 300 A porosity, Alltech Assoc., Inc., Deerfield, IL) equilibrated in buffer A. The column was monitored for absorption at 215 nm; buffer B was 0.1% trifluoroacetic acid, 50% acetonitrile. The flow rate was 1 ml/min and the gradient used was time 0, 0 %B; time 1 min, 0 %B; time 24 min, 70%B; time 25 min, 70%B. 1-ml fractions were collected, dried under vacuum at 50 °C, and were reconstituted in 0.1 ml buffer B to amino acid composition analysis. Also 5 μl of the individual, reconstituted fractions from the peptide maps were mixed with 5 μl of 5 mg/ml bovine serum albumin in 50 mM sodium borate, pH 9, and 5 μl of 80 μg glutaraldehyde. After 5 min, 185 pl of water was added, and the samples were then blotted onto nitrocellulose and probed with avidin-horseradish peroxidase and stained. The results obtained from this approach agreed with those obtained from the first approach.

**Characterization of Sites of Biotinylation**—The site of biotinylation of the various probes was investigated using peptide mapping and the results are summarized in Fig. 2. These results show that melittin was biotinylated primarily on a single peptide which elutes at 12 min indicated by the arrow in the figure. This peptide was completely depleted when avidin was added to the digest. The amino acid composition of this peptide was consistent with sequence positions Val²–Arg² of melittin. An N-hydroxysuccinimidyl ester such as the biotin analogue would be expected to react with aliphatic amines or sulfhydryls. Melittin (and also calmodulin) lacks cysteine. Thus, it was presumably the α-amino moiety of Lys⁵ of melittin which was biotinylated.

BC2 also gave a very simple labeling pattern. A single peptide (indicated by the arrow in the figure), eluting at 11.6 min, is biotinylated. The site of labeling for BC2 has been reported to be Lys⁴ (27), and our amino acid composition data are consistent with this. BC1, however, has a very complex labeling pattern. At least four different peptides (denoted by arrows at 15.5, 18.1, 19.5, and 21.7 min) are depleted by the addition of avidin. Adding twice as much avidin depletes these further (data not shown). For so complex a labeling pattern, we did not attempt to purify and analyze these peptides further. Kincaid et al. (26) have reported that the conditions used result in the incorporation of 2 equivalents of biotin into calmodulin but, as was pointed out by these authors, the method used is of uncertain accuracy (26). The sites of labeling were not reported. Assuming 2 equivalents, the results from these peptide mapping studies suggest that BC1 represents a heterogeneous population of calmodulins biotinylated at several different sites with an average stoichiometry for the population of about two.

**RESULTS**

MLCK, CN, and phosphorylase b kinase are well characterized calmodulin-activated enzymes. As shown in Fig. 3, they also bind biotinylated calmodulins, melittin, and the anti-melittin antibody. The calmodulins used for probing were present at 250–270 nm while the melittin was 500 nm. Thus, the interaction with these probes occur at low concentrations. 1 μg of BC1 and 1 μl of the anti-melittin were blotted in the upper two wells to provide positive and negative controls to the experiments. Notice that the blotted anti-melittin bound the biotinylated melittin probe as would be expected. The anti-melittin used to probe the enzyme blots was a 1:10 dilution, and lower amounts do not detectably bind to some of the enzymes such as CN. MLCK had been previously reported (21) to bind the anti-melittin antibody as was seen in Fig. 3; binding to the other enzymes has not previously been reported. This experiment (Fig. 3) shows that there are some basic similarities between these calmodulin-activated enzymes. They all bind calmodulin, and other experiments show that this binding occurs only in the presence and not in the absence of Ca²⁺ (i.e. in EGTA). As predicted by the model, they also all possess both a melittin-like region recognized by the antibody and a binding-site for melittin detected by BM. Both of these latter interactions were found to occur in EGTA or Ca²⁺, i.e. they are Ca²⁺-independent. This is consistent with the model since Ca²⁺ binding and thus Ca²⁺ dependence of binding is a property of calmodulin. Fig. 3 also shows some evidence for diversity within this group of enzymes. While all of the enzymes bind BC1 well, phosphorylase b kinase does not bind BC2 well. However, phosphorylase b kinase does bind both melittin and anti-melittin better than the other enzymes. CN binds melittin better than MLCK but binds anti-melittin less well than does MLCK. Thus, while all the enzymes are similar in being able to bind all the probes, how well they bind the individual probes is different for individual enzymes. Since BC1 is apparently a heterogeneous population of biotinylated calmodulins (Fig. 2), this may explain the success of BC1 at binding well to so many of the calmodulin-binding proteins (see also below); one or more of the biotinylated species in BC1 can bind to any of the enzymes. In contrast, BC2, because it represents a monolabeled derivative, is only bound with high affinity by those enzymes unaffected

![Fig. 2. Peptide maps of the biotinylated melittin and calmodulins](image)

**Fig. 2. Peptide maps of the biotinylated melittin and calmodulins.** Trypsin digests of the biotinylated probes with or without the addition of avidin were resolved using the high pressure liquid chromatography peptide mapping procedure described. All of the maps are shown to the same scale. The arrows indicate the position of peaks which were depleted by the addition of avidin. Fractions of 1 min were also collected, dried, and probed for biotin and subjected to amino acid composition analysis.

![Fig. 3. The binding of the four probes to myosin light chain kinase (MLCK).](image)

**Fig. 3.** The binding of the four probes to myosin light chain kinase (MLCK). Calcineurin (CN), and phosphorylase b kinase (PbK). The amounts of enzyme spotted on the nitrocellulose was chosen to give approximately equal staining with biotinylated calmodulin 1 (BC1). These were MLCK, 0.3 μg; CN, 1 μg; phosphorylase b kinase, 10 μg. As controls, 1 μg BC1 and 1 μl antimelittin antibody (AM) were also spotted. The blots were probed in the presence of 1 mM CaCl₂ and detection was with avidin- (the leftmost three) or goat antirabbit IgG-horseradish peroxidase (the rightmost conjugates as appropriate.)
by the Lys84 modification.

The specificity of the binding of melittin to calmodulin-binding proteins was next investigated using gel blot experiments. Fig. 4 shows gel blots of two crude tissue extracts (rat brain and Arabidopsis) probed with the biotinylated calmodulins, melittin, or the antimelittin antibody. The results are further analyzed in Table I.

The large amount of protein (100 μg) used in these blots of minigels gives a very dark smear of protein when stained in the normal manner with Amido Black stain, and short exposures to the stain (5 min) were used so that some detail could be observed. Contrasting the Amido Black staining pattern with that obtained with the various probes reveals that most of the proteins present in either tissue extract do not bind any of the probes. Thus, the binding of melittin, anti-melittin, or the calmodulins is highly specific for a relatively small number of proteins in both tissues.

Furthermore, there is a close correspondence between the proteins which bind calmodulin and those which bind melittin and its antibody. For Table I, the molecular weight standards were used to calculate a molecular weight for each of the proteins which bound the probes. Because of small differences in protein migration in the individual blots, those bands which occur within 1 mm of each other are grouped together. The results show that while BC1 and BC2 bind to many of the same bands, the relative intensity of staining differs in several cases indicating that some bands bind one biotinylated adduct of calmodulin better than the other. BC1 and BC2 also clearly bind to some proteins which do not bind the other probes. This is most clearly seen with the two highest molecular weight bands observed in the brain extract. These two bands probably represent the two subunits of fodrin (also called calpspectin), a calmodulin-binding protein abundant in brain. Fodrin is known to have a relatively low affinity for calmodulin, and this low affinity may account for its failure to bind the other probes. Clearly, melittin and antimelittin also bind to some proteins which do not bind the calmodulin probes. For example, anti-melittin and melittin both bind to a protein in Arabidopsis with a mobility of 47 mm which binds neither BC1 or BC2. Clearly, anti-melittin detects the fewest bands, probably because of the weak cross-reactivity between this antibody and the proteins present in the extract. At the highest concentration of anti-melittin used, only those proteins with the strongest cross-reactivity are observed. However, in spite of the several differences found, the close correspondence between calmodulin, melittin, and anti-melittin binding in both tissues is remarkable. Most proteins which bind calmodulin also bind melittin (Table I) and many cross-react with an anti-melittin antibody (Fig. 4).

It is also clear from Table I and Fig. 4 that these probes are detecting considerable diversity within the group of proteins which bind the probes. For example, in the brain extract, the most intense staining occurs with BC1, BC2, BM, and AM for proteins with mobilities of 15, 3, 16.5, and 28 mm, respectively. This diversity was also apparent in the results presented in Fig. 3.

Several control experiments relate to the experiment in Fig. 4. In the brain extract, the band seen just above the 14,000 molecular weight standard (Fig. 4) was also observed in blots when no biotinylated probe was used, indicating that it is artifactual. When higher concentrations of biotinylated melittin were used other bands were detected, but at low concentrations a specific pattern (Fig. 4) of stained bands is consistently observed. As had been observed in other experiments, the binding of BC1 and BC2 was found to be Ca2+-dependent while that of BM and AM was Ca2+-independent. Finally, when 10 μM melittin (not biotinylated) was added to the antimelittin antibody prior to its use in probing the gel blots, no binding of anti-melittin was observed, indicating that it is the melittin binding property of the antibody which accounts for the staining observed.

A crude extract was also prepared from pea seedlings, fractionated by gel electrophoresis, and probed with BC1, BC2, and BM (data not shown). The result was similar to that found with the other tissue extract. The majority of the proteins which bind the calmodulins also bind melittin and again, the comparison of staining intensities obtained with each probe showed evidence for diversity within this group of proteins.

The gel blot experiments (Fig. 4) argue strongly for the specificity of melittin's binding; melittin does not bind to most protein but rather binds selectively to proteins which also bind calmodulin. Other experiments also support this specificity. In one such experiment, phosphorylase b kinase, phosphodiesterase, NAD kinase, and three tissue extracts (pea, Arabidopsis, and rat brain) were blotted onto nitrocellulose and probed with 0.5 μM BM containing different concentrations of poly-L-lysine (average molecular weight 3900). Even 100 μM polylysine did not affect the amount of BM

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**Figure 4.** Gel blots of rat brain (RB) or Arabidopsis (AR) crude tissue extracts. 100 μg of the crude extract was resolved using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted over to nitrocellulose. The blots were then either stained with Amido Black or blocked with bovine serum albumin and probed with the biotinylated calmodulins (BC1 and BC2, both at 250 nM), melittin (BM, 150 nM), or the antimelittin antibody (AM, 1:10) in the presence of 1 mM CaCl2. Detection was with the alkaline phosphatase conjugates. The results are summarized in Table I.

**Table I**

| Mobility (mm) | Comparison of gel blot results with three of the probes |
|--------------|--------------------------------------------------------|
|              | Rat brain | Arabidopsis |
|              | BC1 | BC2 | BM | BC1 | BC2 | BM |
| 1 ~200        | +   | +   | +  | +   | +   | +  |
| 3 ~200        | +++ | +++ | +++| +++ | +++ | +++|
| 13            | 75  | +   | +  | +   | +   | +  |
| 15            | 66  | +   | +  | +   | +   | +  |
| 16            | 64  | ++++| +  | +   | +   | +  |
| 17            | 62  | +   | +  | +   | +   | +  |
| 19.5          | 58  | +   | +  | +   | +   | +  |
| 21            | 54  | +   | +  | +   | +   | +  |
| 22.5          | 51  | +   | +  | +   | +   | +  |
| 23.5          | 49  | +   | +  | +   | +   | +  |
| 27            | 43  | +   | +  | +   | +   | +  |
| 29            | 41  | +   | +  | +   | +   | +  |
| 31            | 38  | +   | +  | +   | +   | +  |
| 34.5          | 34  | +   | +  | +   | +   | +  |
| 36            | 32  | +   | +  | +   | +   | +  |
| 38            | 30  | +   | +  | +   | +   | +  |
| 41            | 27  | +   | +  | +   | +   | +  |
| 44            | 25  | +   | +  | +   | +   | +  |

*Since the highest molecular weight standard used was 97,000, mobilities in this region do not yield accurate molecular weights.
bound by any of the enzymes or extracts relative to the staining observed in the absence of polylysine (data not shown). Thus, it seems unlikely that the basic properties of melittin alone account for the binding observed. Also, in various gel blot experiments the following proteins did not bind biotinylated melittin (or the biotinylated calmodulins): myosin, β-galactosidase, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Interestingly, glycogen phosphorylase b was found to bind to the biotinylated calmodulins and melittin (data not shown). The binding and activation of phosphorylase b by calmodulin has been previously reported (31, 32) although its physiological relevance has been questioned (32). Thus, phosphorylase b is another calmodulin-binding protein which also binds melittin.

The diversity apparent in Figs. 3 and 4 was further investigated in Fig. 5. Fig. 5 presents a dose-response comparison of CN and phosphorylase b kinase using all four probes. All the buffers used in the experiment contained 1 mM Ca++. While both enzymes bind BC1 over about the same concentration range, it is clear that CN has a much higher affinity for BC2 than does phosphorylase b kinase. BC2 has been shown to be biotinylated at Lys^58, while BC1 represents calmodulins biotinylated at several sites. From Fig. 5, it appears that modification of Lys^58 has a rather dramatic effect on the ability of calmodulin to bind to phosphorylase b kinase. For CN, half-maximal binding was observed between 10–100 nM for both BC1 and BC2. For phosphorylase b kinase, half-maximal binding occurs between 100–1000 nM BC1 and above 5000 nM for BC2.

It is also clear from Fig. 5 that anti-melittin binds much more strongly to phosphorylase b kinase than to CN. The most probably explanation for this would be that phosphorylase b kinase possesses a region which is much more similar to melittin or is more accessible to the antibody than the corresponding region on CN. Thus, the results with these three probes further demonstrate the differences between phosphorylase b kinase and CN which are indicative of the diversity among the calmodulin-binding proteins already seen in the other experiments.

It was consistently found that BM is detected much more weakly by the avidin conjugates (with alkaline phosphatase or horseradish peroxidase) than are the other biotinylated probes. The cause for this is unknown but presents no problem to most experiments since staining longer for alkaline phosphatase or horseradish peroxidase can be used to amplify the observed staining with little increase in background. However, in the experiment in Fig. 5, all of the blots were stained for the same amount of time and the result is that BM binding is only barely detectable under these conditions. However, the low background allows some preliminary observations. BM also binds more strongly to phosphorylase b kinase than it does CN showing further the differences between these two enzymes.

In Fig. 6, the binding of melittin to four different enzymes, including phosphorylase b kinase, is shown to occur in EGTA. For this experiment, the blots probed with BM were stained longer although this increased the background somewhat; however, the results are still readily interpretable. The dose response for BM in EGTA is also similar to that observed in other experiments which included Ca^2+ (data not shown). The model predicts that Ca^2+ binding during enzyme activation is a property of calmodulin not the enzymes.

Two other enzymes were also investigated during the course of this study, but the data is not shown. NAD kinase and phosphodiesterase also bind BC1 and BC2 in a Ca^2+-dependent manner and both bind BM and AM in a Ca^2+-independent manner (see also Fig. 6).

To independently confirm that melittin binds to calmodulin-activated enzymes, 0.43 units of phosphodiesterase were applied to a 1-ml melittin-Sepharose column in a Ca^2+-containing buffer. 88% of the applied enzyme bound to the column and no further activity could be eluted by washing the column with EGTA or 6 M guanidinium chloride. In a related experiment, melittin was added directly to a phosphodiesterase assay carried out in 0.2 mM EGTA and in the absence of calmodulin. The small basal activity measured under these conditions was unaffected by melittin concentrations below about 8 μM, but at a higher concentrations the enzyme activity was stimulated by melittin. At 213 μM melittin (the highest concentration tested), the phosphodiesterase activity rose to 21% of the activity obtained with saturating amounts of Ca^2+ and calmodulin. Thus, melittin stimulates phosphodiesterase in a Ca^2+-calmodulin-independent manner at high concentrations (data not shown).

The results presented show that, in agreement with the model proposed, calmodulin-activated enzymes possess 1) a melittin-like region which is bound by the anti-melittin antibody. Since Kaetzel and Dedman (21) have shown that the binding of anti-melittin blocks calmodulin binding, it is likely the enzyme region which binds anti-melittin is also the one which 2) binds the biotinylated calmodulins BC1 and BC2. We will refer to this region as the CBS. These enzymes also possess 3) a melittin-binding site. Because melittin-binding is

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**Fig. 3.** Calcineurin (CN) and phosphorylase b kinase (PbK) have differences in affinity for the probes. 10 μg of each enzyme (indicated on the left of the figure) was blotted for each well and probed with 0.5 ml of biotinylated calmodulins (BC1, BC2), biotinylated melittin (BM), and the antimelittin antibody (AM) as shown on the right of the figure. The nanomolar concentration of the three former probes is shown at the top of the figure and the dilutions of AM are shown at the bottom. Detection was with the horseradish peroxidase conjugates. All solutions contained 1 mM CaCl₂. The rightmost well for CN-BC2 was of lower intensity than expected in this experiment but not in others and is artifactual due to a well that drained poorly during the experiment.

**Fig. 6.** Diversity of calmodulin-activated enzymes is also observed with biotinylated melittin (BM). The four enzymes shown on the right of the figure were blotted and probed with BM. The amounts blotted were calcineurin (CN, 1 μg), phosphorylase b kinase (PbK, 1 μg), pea NAD kinase (NADK, 0.19 μg), and myosin light chain kinase (MK, 0.25 μg). They were probed with the micromolar concentrations of BM shown at the top of the figure. For this experiment, all of the solutions contained 0.1 mM EGTA. Detection was with the avidin-alkaline phosphatase conjugate.
also a characteristic of calmodulin, we refer to this region as the CLBS. Having established that melittin binds to calmodulin-binding proteins, we were curious to see if a binding site could be identified in enzymes of known sequence.

The CBS region of enzymes of known sequence has received a great deal of attention. In Table II 12 putative CBSs that have been identified are presented. Melittin is typical of the CBS sequence identified for these enzymes in that it contains 1) a cluster of basic amino acid residues adjacent to a group of hydrophobic residues which typically include aromatic amino acids like tryptophan, and 2) further down the peptide chain another group of basic adjacent-to-hydrophobic residues is also found. Several investigators (8-17) have pointed out these features of putative CBS peptides. The CBS sequences shown are not homologous in direct identity between matched sequence positions but are homologous in that they share these chemical characteristics. For example, of the 12 CBS peptides shown in Table II, all of them have at least two basic adjacent-to-hydrophobic clusters described above, all have one or more aromatic residues, and nine of the 12 have a tryptophan.

Using these characteristics for CBS peptides, we predicted that CLBS peptides should have the following characteristics: 1) at least two regions of acidic adjacent-to-hydrophobic residues and 2) aromatic residues among the other hydrophobic residues with tryptophan commonly being found. These are rather broad criteria but no more broad than has been used to identify putative CBS peptides. We searched the entire sequences of the calmodulin-binding enzymes of known sequence; the results are shown in Table II. When these putative CLBS and CBS sequences from the same enzyme are aligned, the placement of charged and hydrophobic amino acids is striking. The sequences were aligned by simply placing the largest cluster of charged residues to the left for both chains and aligning the charges with one another. In some cases, the CBS and CLBS sequences were aligned anti-parallel so that the largest charge cluster would be to the left. This alignment should not bias alignments elsewhere along the peptides, and so alignments of hydrophobics and charges elsewhere are due to only the sequence of the peptides and have not been perturbed by introducing gaps or any other feature which might improve the apparent alignment. Neglecting phosphorylase b kinase which will be discussed separately, most of the other sequences show a striking alignment between the CBS and CLBS regions as though these sequences could associate in the native enzymes. The alignment of the positive with negative charges for the two MLCKs and for calmodulin kinase II-β is most striking. The two regions from Ca²⁺-ATPase also align well. The alignment for phosphofructokinase is also good but not as striking as for the others. For these five enzymes, notice that all of the CLBS peptides contain tryptophan and four of the five CBS peptides do. It is also striking that of the four enzymes where tryptophan occurs on both the CBS and CLBS peptides, in three cases, the tryptophans align within three sequence positions of each other.

Several other similarities between the first six enzymes in the table (including the catalytically active phosphorylase b kinase-γ but excluding phosphorylase b kinase-α, phosphorylase b kinase-β, and the non-enzymes melittin and troponin I) are also of interest. In every case, the putative CBS sequence is to the COOH-terminal of the enzyme’s sequence while the putative CLBS is more toward the amino-terminal. In every case, the CLBS regions occur in parts of the sequences which have no postulated role in catalysis or any other known function. For the protein kinases (the MLCKs, calmodulin kinase II-β, and phosphorylase b kinase-γ), a sequence region has been identified which is homologous in all known protein kinases and probably represent the regions of sequence involved in catalysis (8, 10, 13, 17). In all cases, this catalytic region is NH₂-terminal to the CLBS and CBS regulatory sequences. The regulatory sequences are also COOH-terminal to the sequence regions of phosphofructokinase and Ca²⁺-ATPase which are thought to be involved in catalysis (12, 16).

Phosphorylase b kinase was perhaps the most interesting enzyme investigated for Table II in that CBS (13-15) and CLBS regions (Table II) were found for the α, β, and γ subunits. This was further investigated in the experiment in Fig. 7. As can be seen, in 1 mM CaCl₂ BC1 and BM bind to all three subunits while BC2 binds only to the γ subunit. In the absence of Ca²⁺, BC2 no longer binds to γ or any other subunit while BC1 binds weakly to both γ and β but not to α. BM binds to all three subunits in the presence (Fig. 7) or absence (data not shown) of Ca⁺⁺. This is to be contrasted with the behavior of the holoenzyme. The holoenzyme binds BC1 at lower concentrations than it does BC2 (Fig. 5), and the binding is Ca²⁺-dependent.

Surprisingly, the δ-subunit (calmodulin) of phosphorylase b kinase did not stain with BM in Fig. 7. This was further investigated. In NAD kinase assays in which 1 nM calmodulin was included, 50% inhibition occurred between 3-4 nM for both melittin and BM (data not shown). Since melittin is presumably inhibiting by binding to calmodulin in these assays (see below), biotinylation of melittin apparently has little effect on its calmodulin affinity. Rather the results in Fig. 7 apparently arise from two different phenomena. 1) Calmodulin does not bind very well onto 0.45-μm pore nitrocellulose (notice the low intensity of the Amido Black stain of the δ-subunit, Fig. 7); 2) Calmodulin blotted onto nitrocellulose does not detectably bind BM very well (data not shown). The reason for this last phenomenon is not known but may be due to the binding of calmodulin to nitrocellulose interfering with the calmodulin-biotinylated melittin interaction or its detection with avidin-enzyme conjugates.

**DISCUSSION**

Here, we found that five calmodulin-activated enzymes (CN, MLCK, phosphorylase b kinase, phosphodiesterase, and NAD kinase) bind not only calmodulin but also melittin as predicted by the model in Fig. 1. This was shown by using five purified enzymes and by examining the correspondence between melittin and calmodulin binding in two crude tissue extracts. Glycogen phosphorylase b was also shown to share this behavior. Eight sequences of calmodulin-binding proteins or subunits were examined, and the region corresponding to the CLBS which may bind tais melittin could be identified based upon its predicted chemical characteristics.

We also found considerable evidence for diversity within the calmodulin-activated enzymes in the binding of these ligands. This was observed with calmodulins which differ only in their sites(s) of biotinylation and with the other probes. To consider only the results with two of the enzymes, CN binds BC1 and BC2 with similar affinity. In contrast phosphorylase b kinase does not bind BC2 very well and requires at least 10 times as much of this calmodulin to show detectable binding (Fig. 5). Similar evidence for diversity comes from the other probes; phosphorylase b kinase binds both biotinylated melittin and the anti-melittin antibody at lower concentrations than does CN (Figs. 2, 5, and 6). Because of this diversity found among well characterized enzymes, it was necessary in investigating the crude tissue extract gel biotin (Fig. 4) to stain
## TABLE II

Comparison of putative binding domains in calmodulin-binding proteins

Sequences are shown aligned as described in the text. Symbols used: +, - to indicate charged amino acids; * indicates hydrophobic aliphatic; and "A" indicates aromatic. Colons (:) show the position of the first and last charge in melittin and the position of its Trp to aid in showing alignment, and Trp (W) is underlined in all sequences. CaM K II, calmodulin kinase II; PFK, phosphofructokinase.

| Protein          | Type | Alignment |
|------------------|------|-----------|
| **Melittin**     | CBS  | QQRKKNISILAPLGLTLTVKVLAVAGIG     |
| (26-1)           |      | +++*A++ **+** ++**+** A+*++ *   |
| **TN-I**         | CBS  | KLMADASMRVRLPFPKFGRDLFLKQN       |
| (123-96)         |      | ++* -- *+*++ ++A+ +--A++*       |
| **MLCK skeletal**| CBS  | KERKMKKNFIAYSAANRPKCISSGALM     |
| (677-600)        |      | +++A++ A+* +A++* *           |
| **MLCK skeletal**| CLBS | PFLGDDDTETLNSGLNHYDEETFAPSD   |
| (492-522)        |      | A+ -- -- *  ** AAA-- A-- A   |
| **MLCK, gizzard**| CBS  | RMKCMARRRQWQKTHGHAIRPPAPKSGYTVGM|
| (487-512)        |      | ++*+A+ +++A + +* +*+   |
| **MLCK, gizzard**| CLBS | ADDSIEDFAEDDFDMTASTVANNEDEG   |
| (443-415)        |      | --- -- A- -- A- *  ** A+ +**-A |
| **PFK**          | CBS  | KLRGKSNMVWIEYKLHAIHRPPAKPSGSYTVAM|
| (371-405)        |      | ++*+ A+ A-A-+** +A  *+          |
| **PFK**          | CLBS | DEMNDPPPCPIFYKDAQ5SLTVLALYCHGRGMVELVF |
| (240-201)        |      | --- -- A- -- A- *  ** A+ +**-A |
| **CaM K II-α**   | CBS  | KKFNARQLKGAIIITMLATRNPFSVGR         |
| (292-318)        |      | ++++*++ **+** ++* A+  *+        |
| **CaM K II-β**   | CLBS | FDQDKBVGKLY-decoration |
| (214-239)        |      | AA--- +*A + A-A- --A-             |
| **Ca2+-ATPaseα** | CBS  | RELRRGQILWFRGLNRGQRTVQRVAFFSLYEG |
| (1,098-1,131)    |      | +**+ **AA+* --** **AA+* A*        |
| **Ca2+-ATPase**  | CLBS | EPEPEWENDIVTGLCTIADVGIEPD         |
| (659-685)        |      | --- -- A- -- ** A+ ** A+ *---     |
| **Pb K-γ**       | CBS  | GKKFVICTVLSVRIYQQYRRVKP          |
| (294-333)        |      | ++*+ A* ++ A+ A++ A+          |
| **Pb K-γ**       | CLBS | EEVYQIFPHALAEATRYKQRQVQLRSLKDVKVTDSTD |
| (296-250)        |      | --- -- A+ AA -- A+ ** A+ +**-A -- |
| **Pb K-α**       | CBS  | LGYKGVKIRHGGLIRYGSLTLRRKVEAL     |
| (813-840)        |      | *A+ *+++ A *++A* **++A--*      |
| **Pb K-α**       | CLBS | LYSVDYDDNYDELSGDNMDG            |
| (627-648)        |      | *A --A- A-- A-- A+ --A- A      |
| **Pb K-α**       | CBS  | RQQQMRRRRLGMLRVP1GFYKVWMVKLQK   |
| (1,063-1,095)    |      | ++**+ A+ ** A+ A+ A++A*       |
| **Pb K-α**       | CLBS | FENIECEWLPNTYFILDGVI-SGNAEQVQYREALEAVLI |
| (321-360)        |      | A- *-- A- *AA AA**-- A* A- -- A+-- **A- ---|
| **Pb K-β**       | CBS  | RLYRRAGSKKLAVRSGAAPTQKSFSSS     |
| (767-794)        |      | ++* A+ ++*A+ ** A A+ A      |
| **Pb K-β**       | CLBS | ISITEELPEFKSFEELE            |
| (672-688)        |      | * -- -- A+ A- --A-        |
| **Pb K-β**       | CBS  | NGRCMNLKQRIDGSLRNPFTCRFGVRWQILR |
| (919-950)        |      | A+ *+ *- A A++*A**++     |
the blots in such a way that even minor binding species could be observed. This was necessary because a band which gives a strong signal with one probe may give a much weaker signal with a different probe. When the blots are handled in this way, it is found that most bands which bind calmodulin also bind melittin and many bind the anti-melittin antibody.

The results presented are consistent with previous observations. The major bands detected with BC1 in brain extracts (Fig. 4) had been previously reported (26) and are in agreement with Fig. 4. Several minor proteins not previously reported were also observed because of the longer staining used to optimize detection. Kaetzel and Dedman (21) had previously shown that the anti-melittin antibody binds to calmodulin-binding proteins, including MLCK as was observed here, and that its binding prevents calmodulin binding. We have extended these observations to several other well-characterized enzymes and to two tissue extracts.

The apparent affinities of the calmodulin-binding proteins for the biotinylated calmodulin probes (Figs. 5 and 6) are not very similar to the calmodulin affinities previously reported. This is likely due to the limitations of the blotting experiments. For example, in the experiment in Fig. 5, the 10 μg blotted on the paper is about 10⁻⁶⁹ mol for CN and about 10⁻¹⁰ mol for phosphorylase β kinase. The 0.5 ml of 100 nM BC1 used to probe these enzymes contained only 5 x 10⁻¹ⁱ mol of calmodulin. Clearly, the large amount of enzyme relative to calmodulin used in such experiments would lead to a decrease in the apparent affinity. For this reason, it is not surprising the affinity measured for the biotinylated calmodulins in the blotting experiments presented here are not very similar to the known binding affinity of these enzymes for calmodulin measured using enzyme assays and other techniques. We have been cognizant of this effect throughout and have restricted our experiments to comparisons unaffected by it. The perturbation caused by enzyme concentration does not alter the fact that CN binds BC1 and BC2 about equally well while phosphorylase b kinase binds preferentially BC1 indicating that Lys⁴⁴ must be important to the way in which phosphorylase b kinase binds calmodulin. The high concentration of target enzymes also does not alter the interpretation that anti-melittin binds better to phosphorylase b kinase than it does CN.

The apparent affinity for biotinylated melittin is also probably underestimated for the reason given above and for an additional reason: If the model proposed in Fig. 1 correctly describes the situation, the putative CLBS that binds melittin is bound to the intrinsic CBS region in the non-activated enzyme (i.e. in the absence of calmodulin). Thus, the binding of biotinylated melittin would represent a competition between extrinsic melittin and the intrinsic CBS for binding to the CLBS. This competition would lower the apparent affinity of melittin for the enzymes. Thus, for both of the reasons noted, the apparent affinity of these enzymes for melittin is probably underestimated.

That melittin binds to calmodulin-activated enzymes does not mean that the inhibition of the calmodulin-dependent activities by melittin reported by others (5) is caused by enzyme binding. Calmodulin itself has a nanomolar affinity for melittin, and it is the binding of melittin to calmodulin which antagonizes enzyme activation. In fact, we observed that in the absence of calmodulin, melittin can activate phosphodiesterase, albeit with low affinity.

Many studies involved phosphorylase b kinase for a special reason. Phosphorylase b kinase has two different ways in which it can bind calmodulin. Phosphorylase b kinase is a multisubunit enzyme of about 1.3 x 10⁶ molecular weight and has the subunit structure (αβγδ). The δ subunit is calmodulin and is tightly bound in the holoenzyme even in the absence of Ca²⁺; strongly denaturing conditions are required to remove δ from the complex. Even under some denaturing conditions the catalytic subunit (γ) still binds δ, and isolated γ is activated by calmodulin indicating that δ binds to γ in the holoenzyme. Phosphorylase b kinase can also bind reversibly another four calmodulins in a Ca²⁺-dependent fashion, and these calmodulin are referred as to δ' and δ'' in the holoenzyme (see Ref. 33 for review). Since δ exchanges only slowly with extrinsically added calmodulin while δ′ exchanges readily (34), the BC1 and BC2 binding
shown in Figs. 3 and 5 almost certainly represent their binding as "i". Thus, biotinylation of Lys⁴⁴ of calmodulin (in BC2) has a detrimental effect on "i" binding to the holoenzyme. Since this binding is presumably to α and β (34), one would expect these subunits to bind BC1 better than BC2 and this was in fact observed in Fig. 7; while BC1 binds to both subunits, BC2 does not at the concentration used. Fig. 7 shows that BC1 also stains γ more strongly than does BC2 and suggests that the binding of the β-calmodulin may also be influenced by the biotinylation state of Lys⁴⁴.

Since phosphorylase b kinase has a calmodulin subunit, this complicates somewhat the interpretation of melittin binding to the holoenzyme since melittin binds to calmodulin, and the binding observed with the holoenzyme could represent binding to the β subunit. Two observations argue against this. While high affinity melittin binding to calmodulin is Ca²⁺-dependent (5), the binding to the holoenzyme observed here occurs in either the presence (Figs. 3 and 5) or absence of Ca²⁺ (Fig. 6). Second, isolated α, β, and γ were all shown to bind melittin (Fig. 7), and this binding was also Ca²⁺-independent.

In presenting the results in Table II, phosphorylase b kinase was purposely excluded from much of the discussion. This was done because in such a complex, multisubunit enzyme it is not at all certain that the CBS region(s) of one subunit does not actually bind to a CLBS region on another subunit; in fact, this may be one of the ways these subunits are held together in the holoenzyme. In the other cases discussed, it seems likely that the CBS region would be available for interaction with calmodulin (during activation) or with the intrinsic CLBS sequence (in the non-activated state). For these enzymes, the alignment between CBS and CLBS sequences discussed may be significant but this is not at all certain for phosphorylase b kinase.

Phosphofructokinase may also represent a special case of calmodulin-binding enzymes not adequately described by the model (Fig. 1). This enzyme forms dimers and tetramers and calmodulin affects this oligomerization (11). In such oligomerization, the CBS of one subunit may be binding to the CLBS of another subunit holding the oligomers together.

Irrespective of the subunit-subunit interactions discussed above, it is clear that all calmodulin-activated enzymes we have studied also bind melittin, and a putative CLBS sequence (which presumably binds melittin) can be identified in the enzymes of known sequence. Many of these enzymes are single subunit enzymes, and the existence of these CLBS sites were predicted based on the model in Fig. 1 described to describe calmodulin-activation of enzymes. Melittin-binding and the presence of CLBS on enzymes predicted based on this model appears to be a true feature of these enzymes and thus the model has shown its predictive value.

Melittin was also shown to activate the calmodulin-independent activity of phosphodiesterase, albeit at high melittin concentrations. Such an effect of melittin was also predicted by the model. Many calmodulin-activated enzymes have a small basal activity in the absence of calmodulin. While some of this activity may be due to enzyme activated during isolation by partial proteolysis by tissue proteases, a basal activity has been observed for many of these enzymes even when protease inhibitors have been included throughout the isolation and with enzyme which is homogeneous and shows no evidence of proteolysis. The model in Fig. 1 offers an explanation for this basal activity. In the absence of calmodulin, the CBS region of the enzyme is normally bound by the CLBS region and the enzyme is not active. However, the binding affinity between these two regions is not infinite and, due to thermal motion, some fraction of the time the CBS must dissociate from the CLBS. As long as the CBS is dissociated from the CLBS for a time which is long relative to the half-life of catalysis, the enzyme would be active for this fraction of time. The observation that melittin activates the basal phosphodiesterase activity supports this view. At high melittin concentrations, melittin occupies the CLBS and prevents the CBS from binding; when the CBS is not bound, the enzyme is activated (i.e. no longer internally inhibited).

The model presented in Fig. 1 brings together observations and models from numerous investigators (reviewed in Ref. 2 and further discussed in Ref. 22) into a unified, testable model. A more specialized model has been proposed to account for calmodulin activation of protein kinases, the pseudosubstrate model. This model states that the calmodulin-binding site (here referred to as the CBS) has an amino acid sequence which is similar to the sequence of the site on protein substrates normally phosphorylated by the protein kinase, and the CBS binds to the active center of the enzymes blocking catalysis in the absence of calmodulin. Some of the CBS regions of protein kinases (e.g. smooth muscle MLCK) have a serine in the CBS sequence which is phosphorylatable by other protein kinases, and this phosphorylation activates MLCK and renders its activity calmodulin-independent.

Based upon either model, one would have predicted that melittin (which is similar to the CBS region of enzymes) would bind to calmodulin-activated protein kinases, but the model in Fig. 1 differs in that it predicts the same would be true of enzymes which are not protein kinases, such as NAD kinase, phosphodiesterase, and CN. This model can also account for the activation of phosphodiesterase's basal activity by melittin. The model in Fig. 1 is clearly more general in that it describes both types of calmodulin-activated enzymes, not just protein kinases. There are also, however, other more subtle differences between the two models. In the pseudosubstrate model, the CBS-binding site is also a part of the protein kinases active site while the model presented here places no restriction on the location of the CBS-binding site (i.e. the CLBS). The location of the putative CLBS regions in Table II agrees with this prediction. These sequences all occur in sequence regions which have never been implicated to participate in catalysis. How the model in Fig. 1 compares with another current model, the autoinhibitory model (35, 36), is less certain. The CLBS we have described may correspond to the autoinhibitory region postulated by this model for at least some enzymes. In fact, our location for the CLBS of smooth muscle MLCK (Table II) would be consistent with the general location of the autoinhibitory region described by Ikebe et al. (37). However, our experiments with the activation of phosphodiesterase by melittin (see "Results") suggest that the binding of melittin to the presumptive CLBS of phosphodiesterase can activate rather than inhibit. For this reason, we treat the CLBS here as simply a binding site without ascribing an activating or inhibitory role to it.

Finally, the model in Fig. 1 can account for previously unexplained observations. In their study of synthetic peptides similar in sequence to the CBS of skeletal muscle MLCK, Kennelly et al. (38) showed that several of these peptides could be phosphorylated by MLCK. This was taken as evidence for the pseudosubstrate model. They also studied the ability of these peptides to inhibit proteolytically activated MLCK. The pseudosubstrate model would have predicted these peptides to be simple, competitive inhibitors of catalysis since both the inhibitory peptide and the substrate which is phosphorylated should bind to and thus compete for the same site (i.e. the active site). Based upon the model in Fig. 1, either
non-competitive (i.e., binding to only the CLBS with high affinity) or a mixed type of competitive inhibition would be predicted since the peptide inhibitor could bind both the active site (since it is, after all, phosphorylated) and to the regulatory CLBS site. Mixed, competitive inhibition was in fact observed in these experiments. More recently, Colbran et al. (36) have shown that a peptide (sequence 281–309) containing the putative CBS for type II calmodulin-dependent protein kinase (see Table II) is non-competitive with substrate. This observation is also contrary to the prediction of the pseudo substrate model but would be consistent with the model in Fig. 1.

The model in Fig. 1 is stated concisely to allow its further testing and refinement. Based upon this model, calmodulin activation is viewed as a dynamic competition between calmodulin and the enzyme's CLBS for the CBS region. The enzymes thus flip-flop between an activated and non-activated state depending upon what the CBS is bound to; hence, the name given to the model. If true, the calmodulin affinity of an enzyme is not only a result of how well the CBS "fits" calmodulin but is also influenced by the affinity of the CBS/CLBS pair. The basal (non-calmodulin-dependent) activity of an enzyme can also be modulated by the affinity of the CBS/CLBS pair thus allowing for enzymes with little or no basal activity (e.g., MLCK and NAD kinase) and enzymes where the basal activity is substantial (e.g., erythrocyte Ca2+-ATPase). The model can also accommodate the diversity observed within the group of calmodulin-activated enzymes observed in this and other studies. It has been frequently observed that a given alteration of calmodulin can have a different effect on different enzymes.

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