Localization of Unique Functional Determinants in the Calmodulin Lobes to Individual EF Hands*

Anthony Persechini‡, Paul M. Stemmer‡ and Ichiro Ohashi¶

From the Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York 14642 and the Departments of §Pediatrics and ¶Anesthesiology, University of Nebraska Medical Center, Omaha, Nebraska 68198

We have investigated the functional interchangeability of EF hands I and III or II and IV, which occupy structurally analogous positions in the native I-II and III-IV EF hand pairs of calmodulin. Our approach was to functionally characterize four engineered proteins, made by replacing in turn each EF hand in one pair by a duplicate of its structural analog in the other. In this way functional determinants we define as unique were localized to the component EF hands in each pair. Replacement of EF hand I by III reduces calmodulin-dependent activation of cerebellar nitric oxide synthase activity by 50%. Replacement of EF hand IV by II reduces by 60% activation of skeletal muscle myosin light chain kinase activity. There appear to be no major unique determinants for activation of these enzyme activities in the other EF hands. Replacement of EF hand III by I or IV by II reduces by 50–80% activation of smooth muscle myosin light chain kinase activity, and replacement of EF hand I by III or II by IV reduces by 90% activation of this enzyme activity. Thus, calmodulin-dependent activation of each of the enzyme activities examined, even the closely related kinases, is dependent upon a distinct pattern of unique determinants in the four EF hands of calmodulin. All the engineered proteins examined bind four Ca$^{2+}$ ions with high affinity. Comparison of the Ca$^{2+}$-binding properties of native and engineered CaMs indicates that the Ca$^{2+}$-binding affinity of an engineered I-IV EF hand pair and a native I-II pair are similar, but an engineered III-III EF hand pair is intermediate in affinity to the native III-IV and I-II pairs, minimally suggesting that EF hands I and III contain unique determinants for the formation and function of EF hand pairs. The residues directly coordinating Ca$^{2+}$ ion appear to play little or no role in establishing the different Ca$^{2+}$-binding properties of the EF hand pairs in calmodulin.

The crystal structure of the multifunctional regulatory protein, calmodulin (CaM), 1 shows an elongated molecule in which two globular lobes are joined by an extended central helix (1). Each globular region is composed of a pair of EF hand Ca$^{2+}$-binding domains, which include all amino acid residues in CaM apart from residues 1–8 at the N terminus and 76–81 in the central helix (2). We shall refer to these regions as the N-terminal leader and the central helix linker, respectively. CaM appears to recognize in its enzyme targets basic amphiphilic helix-forming segments that are generally termed CaM-binding domains; there is no consensus amino acid sequence for these regions (3–5). The simplest model for CaM-dependent enzyme activation is one in which the CaM-binding domain also functions as an intrasteric inhibitor whose influence is relieved when it is bound by CaM. In a more complex model, occupancy of the CaM-binding domain is insufficient for enzyme activation and further, secondary, CaM-target interactions are required. Data for CaM-dependent activation of myosin light chain kinase activity suggest that this latter model may apply, while much of the available data for CaM-dependent activation of nitric oxide synthase and cyclic nucleotide phosphodiesterase activities are consistent with the former (6–11).

High resolution structures have been determined for the complexes between CaM and peptides representing the CaM-binding domains in either skeletal or smooth muscle myosin light chain kinase or in CaM-dependent protein kinase II (12, 13). Given the considerable internal structural homology seen in CaM, it is perhaps not surprising that these complexes each exhibit an approximate two-fold axis relating the N- and C-terminal CaM lobes (12, 13). However, in spite of the homology between the two EF hand pairs, the lobes are functionally distinct with respect to Ca$^{2+}$ binding and enzyme activation (8, 9, 14–17).

Definition of the functional determinants unique to each CaM lobe is central to any detailed understanding of CaM function. We have previously investigated the functional interchangeability of the EF hand pairs in CaM (8, 9). The functions of the structurally distinct N-terminal leader and central helix linker sequences also have been investigated in some detail (11, 18–21). In this study, we localize unique functional determinants within the EF hand pairs to the individual EF hand domains.

MATERIALS AND METHODS

Engineered CaMs containing EF hand substitutions were generated using an overlap-extension polymerase chain reaction procedure as described previously (8). The concentrations of purified engineered

* This work was supported by Public Health Service Grant DK44322 and National Heart Association Grant 93008090 (to A. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence should be addressed: Dept. of Pharmacology and Physiology, University of Rochester Medical Center, 821 Elmwood Ave., Box 642, Rochester, NY 14642. Tel.: 716-275-3087; Fax: 716-461-3259; E-mail: np26crocus.medicine.rochester.edu.

¶ The abbreviations used are: CaM, calmodulin; nNOS, rat cerebellar nitric oxide synthase; smMLCK, avian smooth muscle myosin light chain kinase; smMLCK, avian smooth muscle myosin light chain kinase; smMLCK, avian smooth muscle myosin light chain kinase; smMLCK, avian smooth muscle myosin light chain kinase; CaMCC, CaM residues 9–75 are replaced with a duplication of CaM residues 82–148; CaMNN, CaM residues 82–148 are replaced with a duplication of CaM residues 9–75; CaM32C, residues 9–42 are replaced by a duplication of residues 82–115; CaM14C, residues 43–75 are replaced by a duplication of residues 116–148; CaM32C, residues 116–148 are replaced by a duplication of residues 43–75; CaM14N, residues 82–115 are replaced by a duplication of residues 9–42; CaMP1, the changes L116P and E139P have been made; CaMP2, the changes P43L and P66E have been made.

A unique functional determinant is defined as any amino acid or group of amino acids in one CaM EF hand pair that cannot be functionally replaced by its structural analog in the other pair.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
CaMs were determined from their optical absorbances in the presence of 50 μM CaCl₂ using extinction coefficients derived from quantitative amino acid composition data: $E_{276}^{1%} = 0.31$ for both CaMN32 and CaMN14, $E_{276}^{1%} = 0.26$ for CaM14C, and $E_{276}^{1%} = 0.20$ for CaM32C. Previously published extinction coefficients were used for native CaM, CaMCC, and CaMNN (8, 14). For Ca²⁺-binding studies, it was necessary to determine extinction coefficients for engineered CaMs decalcified as described previously (8). These all are within 10% of the values determined in the presence of 50 μM CaCl₂, except the $E_{276}^{1%}$ values for CaMN32 and CaMN14, which are 0.15 and 0.14, respectively.

Rabbit skeletal muscle myosin light chain kinase (skMLCK) was expressed in Sf9 cells using a recombinant baculovirus and purified as described by McMillan et al. (23). Avian smooth muscle myosin light chain kinase (smMLCK) purified as described by Herring (24) and the recombinant baculovirus used for expression of skMLCK were gifts from the laboratory of James T. Stull.

Data for activation and inhibition of CaM-dependent enzyme activities were measured at 25 °C in the presence of 200 μM CaCl₂. These data were analyzed according to single-site activation or competitive inhibition models as described previously (8). All enzyme activities are expressed relative to the maximal enzyme activities measured in the presence of 100 nM CaM, which are ~300 nmol/min/mg protein for nNOS and ~20 μmol/min/mg protein for skMLCK or smMLCK.

Ca²⁺ binding by native and engineered CaMs was measured at 25 °C using the flow dialysis technique as described in detail by Stemmer and Klee (16). The buffer used for these experiments contained 100 mM KCl, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5, with variable amounts of added Ca²⁺. The concentration of engineered or native CaM in each experiment was 20 μM. Data were corrected for loss of Ca²⁺ ion from the dialysis chamber during the experiment (16), and derived values for free [Ca²⁺] and mol of Ca²⁺ bound/mol of CaM were fit to the Adair-Klotz equation with all parameters adjustable.

$$Y = \frac{N}{4} \left( \frac{x}{K_1} + \frac{x^2}{K_1 K_2} + \frac{x^3}{K_1 K_2 K_3} + \frac{x^4}{K_1 K_2 K_3 K_4} \right)$$

(Eq. 1)

The derived values were also fit to an equation describing cooperative binding to two pairs of Ca²⁺-binding sites.

$$Y = \frac{N}{2} \left( \frac{x}{K_1} + \frac{x^2}{atK_1} \right) + \frac{x^3}{K_2 + \beta K_2^2}, \quad 1 - \frac{x^2}{atK_1}$$

(Eq. 2)

$N$ is the number of Ca²⁺-binding sites/mole of native or engineered CaM, $x$ is the concentration of free Ca²⁺ ion, and $a$ and $b$ are interaction factors, $y$ is the number of moles of Ca²⁺ bound/mole of CaM, and $K_i$ through $K_4$ are dissociation constants. Data for Ca²⁺ binding to CaMCC and CaMNN were fit to Equation 2 with $K_1 = K_2$ and $a = b$; the remaining native and engineered CaMs were fit to Equation 2 with parameters for one lobe fixed to values determined from these initial fits (Table I). Least-squares fits of Ca²⁺-binding values to equations were performed using the Prism (GraphPad, Inc.) software package.

**RESULTS**

The structures of the four engineered CaMs used in this study are schematically represented in Fig. 1. As shown in the figure, junctions in the interconnecting loops between the EF hand pairs and between the EF hands and the leader and linker sequences in CaM were utilized in constructing intramolecular substitutions between the EF hand pairs. Two additional engineered proteins constructed for this study are CaMP1, in which the changes L116P and E139P have been made, and CaMP2, in which the changes are P43L and P66E.

In the CaM-nNOS complex, the C-terminal EF hand pair in CaM can be functionally replaced by the N-terminal EF hand pair, so it is not surprising to find that $K_{act}$ and $F_{act}$ values for

![Fig. 1. Schematic representations of CaMN32, CaMN14, CaM14C, and CaM32C. EF hand domains are represented by heavy lines and are numbered according to their relative positions in the native protein, which is schematically represented in the figure. The N-terminal leader (residues 1–8), central helix linker (residues 76–81), and loops connecting the EF hand pairs are indicated by lighter lines. The junctions used in construction of the chimeric proteins are indicated by dashed lines with the junctional amino acids numbered in the native protein scheme.](image-url)
activation of nNOS activity by CaMN32 and CaMN14 are not significantly different from the values determined with native CaM (Fig. 2; Table I). In contrast, the N-terminal EF hand pair cannot be replaced by the C-terminal pair in the CaM-nNOS complex (Table I). Data for activation of nNOS activity by CaM32C and CaM14C indicate that EF hand domain I contains the major unique determinants for activation of this enzyme activity by CaM, although domain II appears to contain additional minor unique determinants (Fig. 3; Table I). Since CaMNN activates skMLCK activity nearly as well as native CaM, it is not surprising to find that CaM32C and CaM14C are good activators of this enzyme activity (Fig. 3; Table I). The poor activation of skMLCK activity seen with CaMN32 suggests that EF hand domain IV contains the major unique determinants for activation of skMLCK activity (Fig. 2; Table I). The dependences of skMLCK and smMLCK upon unique determinants in the C-terminal lobe are qualitatively similar (Table I). However, CaM14C and CaM32C both fail to activate smMLCK activity, indicating a marked dependence upon unique determinants in both N-terminal EF hand domains. This contrasts sharply with skMLCK activity, which is essentially fully activated by CaM14C or CaM32C (Table I). Furthermore, EF hand domain IV contains significant unique determinants for smMLCK binding; none of the EF hands contains unique determinants having a major effect on binding of nNOS or skMLCK to CaM (Table I). Although CaMNN is an extremely poor activator of both smMLCK and skMLCK activities, they are significantly activated by both CaMN14 and CaMN32 (Table I). Similarly, CaMCC fails to activate nNOS activity, but both CaM14C and CaM32C significantly activate this enzyme activity. To a good approximation these observations can be explained by additivity in the effects of single EF hand replacements, although when the properties of CaMs containing single and double EF hand replacements are compared, a significant degree of synergy is also indicated (Table I).

We have investigated the role of the two lone proline residues at positions 43 and 66 in EF hand II of CaM. We have found that neither these prolines nor the corresponding residues, Leu-116 and Glu-139, in EF hand IV are unique determinants.

### Table I

|                     | skMLCK |         |         | smMLCK |         |         | nNOS   |         |
|---------------------|--------|---------|---------|--------|---------|---------|--------|---------|
|                     | $K_{\text{act}}$ (nM) | $K_i$ (nM) | $F_{\text{act}}$ | $K_{\text{act}}$ (nM) | $K_i$ (nM) | $F_{\text{act}}$ | $K_{\text{act}}$ (nM) | $K_i$ (nM) | $F_{\text{act}}$ |
| CaMNN               | 2      | <0.15   |         | 10     | <0.15   |         | 8      | 1       |         |
| CaMN14              | 2.6    | 0.87    | 5.2     | 10     | 0.53    | 1.7     | 1      | 1       |         |
| CaMN32              | 5.7    | 0.36    | 12      | 40     | <0.1    | 4.4     | 2.2    | 0.45    |         |
| CaMCC               | 3.5    | 0.75    |         | 71     | <0.1    | 2.2     | 1.1    | 1.2     |         |
| CaM14C              | 2.1    | 0.98    |         | 71     | <0.1    | 4.4     | 2.2    | 0.45    |         |
| CaM32C              | 1      | 0.97    |         | 1      | 0.45    | 1.1     | 1      | 1.2     |         |
| CaMP1               | 1      | 1       | 1.2     | 0.78   |         | 7       | 0.82   |         |         |
| CaMP2               | 1      | 1       |         |        |         |         |        |         |         |

* Data are taken from Ref. 8 and are shown here for comparative purposes. $K_{\text{act}}$ values for native CaM are ~1 nm for all three enzymes.
for CaM-dependent activation of skMLCK activity (Table I). Based on the properties of CaMP2, we find the EF hand II prolines to be minor unique determinants for activation of both smMLCK and nNOS activities (Table I). The properties of CaMP1 indicate that Leu-116 and/or Glu-139 are major unique determinants for activation of smMLCK activity (Table I). Either or both of these residues also appear to be minor determinants for activation of nNOS activity, insofar as our data suggest that CaMP1 is a better activator of this enzyme activity than native CaM (Table I).

The Ca$^{2+}$-binding properties of CaMN32, CaM32C, CaM14C, CaMN14, CaMNN, and CaMCC were determined; the enzyme activation properties of the latter two of these proteins have been previously described and are summarized in Table I (8). All these engineered proteins were found to bind four Ca$^{2+}$ ions with high affinity, indicating the presence of four functional EF hands (Table II). Ca$^{2+}$-binding values for CaMNN and CaMCC are fit well by Equation 2 with $K_1$, $K_2$, $\alpha$, and $\beta$ fixed to the values obtained for CaMCC (Table II). The values for Ca2$^{+}$ binding to native CaM can be adequately fit using Equation 2 with $K_1$ fixed to the values determined with CaMNN or CaMCC (Fig. 4). Furthermore, the best fits thus obtained produced substantially different fitting parameters for the III-IV EF hand pair in each protein (Table II). Values for Ca$^{2+}$

![Image of graph]

**FIG. 4. Comparison of Ca$^{2+}$ binding to bovine CaM, CaMNN, and CaMCC.** Values for bovine CaM (■), CaMNN (○), and CaMCC (●) are shown along with curves generated according to Equation 1 (solid line) or Equation 2 (dashed line) using the parameters presented in Table II.
binding to these proteins can be fit well using equation 1 (Fig. 6).

**DISCUSSION**

An important approach to understanding structure-function relations in proteins is to study the functional properties of chimeras constructed from functionally distinct homologs. The power of this approach is that it can be used to identify and study those structural features or determinants in a protein that are responsible for its unique functional properties. This method has been applied in investigations of CaM by constructing and evaluating chimeras between CaM and either cardiac troponin C or a yeast calmodulin homolog (7, 10, 25, 26). CaM also contains internal homology between the two EF hand pairs, which have quite distinct properties with respect to both activation of target enzyme activities and binding of Ca$^{2+}$ ion (8, 9, 14–17). Although all four EF hands bind Ca$^{2+}$ and share significant sequence similarity, EF hands I and III or II and IV share the greatest degree of sequence similarity and occupy structurally analogous positions in the EF hand pairs (Figs. 7 and 8). The structural analogy between EF hands I and III and between EF hands II and IV is also evident in the structures of the CaM-peptide complexes that have been determined, where these EF hands are related by an approximate two-fold axis (12, 13). Thus, a comparison only of EF hands I and III or II and IV is relevant to our goal in this study, which was to further define the functional specialization of the CaM lobes. Put another way, it is the differences between EF hands I and III or II and IV, not I and IV or II and III, that define the two EF hand pairs in CaM.

By functionally evaluating the four engineered proteins schematically represented in Fig. 1, we have found that each EF hand domain in CaM contains unique determinants for activation of at least one of three enzyme activities examined. There is a particular concentration of such determinants in EF hands I and IV. We also find that EF hand IV can functionally mimic EF hand II with respect to Ca$^{2+}$ binding, suggesting that EF hand I dominates the Ca$^{2+}$-binding properties of the N-terminal EF hand pair in native CaM. In contrast, EF hand II cannot mimic EF hand IV, although when paired with EF hand III, its Ca$^{2+}$-binding properties approach those of EF hand IV. This...
minimally suggests that EF hands I and III contain unique determinants for the formation and function of EF hand pairs. Residues directly coordinating Ca\(^{2+}\) ion appear to play little or no role in establishing the different Ca\(^{2+}\)-binding properties of the two EF hand pairs in CaM.

Each CaM lobe associates with skMLCK or smMLCK with an apparent dissociation constant of \(\sim 1 \mu M\), and the “effective” concentration of one lobe once the other has bound is \(\sim 1 \mu M\) (9). The consequence of this is that unless the \(K_{act}\) value of an engineered CaM for either of these enzymes is increased by at least 100-fold, there is no reason to suspect a significant change in the degree of association between the lobes of the engineered protein and the enzyme CaM-binding domain. Hence, even with the CaM32C-smMLCK complex, it is unlikely that reduced activation of kinase activity can be attributed to incomplete association of CaM with the CaM-binding domain. Instead, the absence of or reduction in myosin light chain kinase activation seen with several engineered CaMs suggests that occupancy of the CaM-binding domains in the kinases does not necessarily lead to activation of enzyme activity, implying a requirement for secondary CaM-target interactions (6–8, 11, 25–27). Our recent observation that residues in the CaM N-terminal leader sequence contribute significantly to CaM-dependent activation of skMLCK and smMLCK activities provides direct evidence for an involvement of secondary CaM-target interactions in kinase activation, since the leader does not appear to interact significantly with the CaM-binding domains in either of the kinases (3, 11).

It has been shown that the C-terminal EF hand pair can functionally replace the N-terminal pair in activation of skMLCK, but not smMLCK, activity (8). The failure of CaM14C and CaM32C to significantly activate smMLCK activity demonstrates that there are critical unique determinants for activation in both N-terminal EF hands (Table I). In addition, it is evident that EF hand domain I contains significant unique determinants for smMLCK binding by CaM (Table I). It has been demonstrated that the smMLCK and skMLCK CaM-binding domains are functionally interchangeable, and that the apparent affinity of each CaM lobe for its respective binding site is similar for the two kinases (9, 28). The C-terminal half of

---

**Fig. 6. Ca\(^{2+}\) binding to CaMN32 and CaMN32.** Values for CaMN32 are presented in panel A, and values for CaM32C are presented in panel B. Curves generated according to Equation 1 (solid line) or Equation 2 (dashed line) using the parameters presented in Table II are shown. For purposes of comparison, the curves in Fig. 4 generated according to Equation 2 have been superimposed (dotted lines).
the CaM-binding domain in both kinases appears to interact with the N-terminal CaM lobe (13, 29). However, in skMLCK this region is 12 residues from the C terminus of the protein, while in smMLCK it is more than 100 residues from the C terminus (28, 30, 31). This difference may place additional constraints upon CaM-dependent activation of smMLCK activity that are reflected in its greater dependence upon unique determinants in the N-terminal CaM lobe. The effects of individually replacing the C-terminal EF hands in CaM on its ability to activate smMLCK and skMLCK are qualitatively similar, suggesting that the C-terminal lobe may play similar roles in CaM-dependent activation of the two kinase activities. It has been suggested that the initial event in CaM-dependent activation of either kinase activity is the association of the C-terminal CaM lobe with the N-terminal CaM-binding domain (9). The amino acid sequence of this region in the two kinases is characterized by a conserved Trp residue, whose structural analog is solvent-exposed in the crystal structure of CaM-dependent protein kinase I (32). If the Trp in the two light chain kinases is also exposed, this would provide a rationale for the observed ordered binding of the CaM lobes to these proteins.

In contrast with the myosin light chain kinases, nNOS binds the C-terminal lobe of CaM with an apparent affinity that is similar to intact CaM and is several thousand-fold higher than the apparent affinity of the N-terminal lobe (9). Although the N-terminal lobe contributes little to the overall affinity of the CaM-nNOS complex, both lobes must be bound in order for enzyme activation to occur (9). Thus, a reduction in the degree of association of the N-terminal lobe with nNOS caused by engineered changes in CaM could significantly reduce activation of nNOS activity without greatly affecting the overall affinity of the engineered CaM-enzyme complex. Unless this possibility is eliminated, it is unnecessary to invoke secondary CaM-target interactions in order to explain the inability to activate nNOS activity of an engineered CaM that still binds the enzyme well. Interestingly, CaMP1, made by intramolecular homologous replacement of Leu-116 and Glu-139, is actually a better activator of nNOS activity than native CaM (Table I). The enhanced activation of nNOS activity seen with CaMP1 is perhaps due to an increased association between the native N-terminal lobe in CaMP1 and the nNOS CaM-binding domain, possibly the result of alterations in intramolecular contacts between the CaM lobes in the CaMP1-nNOS complex. Su et al. (26) have previously suggested that such intramolecular contacts influence the stability of the CaM-nNOS complex.

Although we constructed four mutant proteins for this study, only two novel EF hand pairs were made: I-IV and III-II (Fig. 1). Three-dimensional models of the native and engineered EF hand pairs are presented in Fig. 7. This figure illustrates the
similarity in the structures of the hydrophobic clefts in the I-II and III-IV EF hand pairs, which allows us to predict with some degree of confidence the structures of the hydrophobic clefts in the novel I-IV and III-II EF hand pairs (Fig. 7). In particular, it is noteworthy that at the entrances to the hydrophobic clefts formed by the native and engineered EF hand pairs are four identically placed methionine residues. These are thought to play an important role in forming the entropically favorable hydrophobic interactions that occur between native CaM and its many target amino acid sequences (33, 34). The aligned sequences of corresponding EF hand domains in the two CaM lobes reveal many identical or highly conserved amino acid positions (Fig. 8). Residues in the hydrophobic clefts of CaM are particularly well conserved. In contrast, there are significant differences between the EF hand pairs in the distribution of charged residues, many of which participate in electrostatic hydrogen bonds with residues in CaM-binding domains (3). This suggests that differences in charged residues may be the dominant factor establishing the differences between the two EF hand pairs in CaM with respect to enzyme binding and activation. Consistent with this, Findlay et al. (35) have recently presented evidence suggesting that electrostatic hydrogen bonds are more important than hydrophobic interactions in establishing the different peptide-binding specificities exhibited by the CaM lobes.

We find that Ca\textsuperscript{2+} binding to CaMNN or CaMCC is adequately described by a model in which the N- and C-terminal EF hand pairs bind Ca\textsuperscript{2+} identically, with positive cooperativity between EF hands in each pair (Table II). The Ca\textsuperscript{2+}-binding properties of CaMNN or CaMCC are consistent, respectively, with the properties of EF hands I-II or III-IV in intact CaM (17, 36–38). Curves generated to fit Ca\textsuperscript{2+}-binding values for CaM, CaMNN, and CaMCC using Equation 2 with parameters for one EF hand pair fixed as specified in Table I, are similar to the curves generated using Equation 1. This supports the validity of the simpler analysis based on Equation 2 (Figs. 4 and 5). The situation with CaMNN2 and CaMCC2 is more complex. It is evident that the Ca\textsuperscript{2+}-binding affinity of a III-II EF hand pair is intermediate to the affinities of the native I-II and III-IV pairs (Fig. 6). Furthermore, our analysis suggests that Ca\textsuperscript{2+} binding to this engineered EF hand pair is affected by its position in the protein. The value of $\beta$ obtained for the III-II EF hand pair in CaMCC3 is 3.5, indicating negative cooperativity in Ca\textsuperscript{2+} binding; a $\beta$ value of −1 is obtained for CaMNN2 indicating no binding cooperativity (Table I). Finally, the significant difference between curves generated using Equation 1 or Equation 2, with parameter values for the native EF hand pairs in CaMCC2 or CaMNN2 fixed, suggests that the III-II EF hand pairs somehow influence Ca\textsuperscript{2+} binding to the native EF hand pair in each of these proteins. Maune et al. (37) have also observed the appearance of significant interlobe effects on Ca\textsuperscript{2+} binding in some engineered CaMs. Mackall and Klee (39) have presented evidence suggesting that such interlobe effects on Ca\textsuperscript{2+} binding are a consequence of Ca\textsuperscript{2+}-dependent changes in the conformation of the central helix.

EF hands II and IV have similar Ca\textsuperscript{2+}-binding properties when paired with EF hand I, but have distinct properties when paired with EF hand III. This minimally suggests differences between EF hands I and III in determinants for the formation and function of EF hand pairs. EF hands I and III are strikingly similar with respect to their hydrophobic surfaces, except at the beginning of the first helix in each, where Ile-9 and Phe-12 in EF hand I are replaced by Glu and Ile residues in EF hand III (Fig. 8). This difference allows EF hand I to form a Phe-Phe or Phe-Tyr aromatic ring interaction when paired with EF hand II or IV, respectively (Fig. 7). There are also differences between EF hand I and III in the number and distribution of charged residues, notably the replacement of Glu-52 and Glu-83 in EF hand III with Ile-9 and Ala-10 in EF hand I (Fig. 8). Linse et al. (40) have shown that surface electrostatics in the vicinity of the Ca\textsuperscript{2+}-coordinating residues can have substantial effects on Ca\textsuperscript{2+} binding to the EF hand pair in calbindin D\textsubscript{9k}. In spite of their apparently similar Ca\textsuperscript{2+}-binding properties, there are also numerous differences between the amino acid sequences of EF hands II and IV, notably the presence of two lone prolines in the first of these (Fig. 8, Table II). The residues directly coordinating Ca\textsuperscript{2+} ion are better conserved between EF hands I and III than between EF hands II and IV; they appear to play little or no role in establishing the different Ca\textsuperscript{2+}-binding properties of the EF hand pairs in CaM (Fig. 8).

Studies are proceeding to establish the relative contributions of differences in hydrophobic and charged residues to the functional specialization of the lobes, both with respect to Ca\textsuperscript{2+} binding and activation of enzyme activities.

Identification of determinants for target activation that are unique to each EF hand pair in CaM allows us to develop structure-function difference maps between the EF hand pairs that define their functional specialization. An understanding of this functional specialization is central to any detailed model for CaM function in the cell. Others have previously investigated the properties of chimeras between CaM and related, but functionally divergent, Ca\textsuperscript{2+}-binding proteins in order to determine structure-function differences (7, 10, 25, 26). While this approach is very informative, it is quite distinct from an intramolecular structure-function difference analysis between the two lobes in CaM, which must function together in a unified mechanism. This type of analysis addresses in the most direct possible way the functional specialization of the lobes.

REFERENCES

1. Babu, Y. S., Bugg, C. E., and Cook, W. J. (1988) J. Mol. Biol. 204, 191–204
2. Persechini, A., Moncrief, N. D., and Kretsinger, R. H. (1989) Trends Neurosci. 12, 462–467
3. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 119–136
4. James, P., Vorherr, T., and Carafoli, E. (1995) Trends Biochem. Sci. 20, 38–42
5. Persechini, A., and Kretsinger, R. H. (1988) J. Cardiovasc. Pharmacol. 12, 81–12
6. VanBerkum, M. F. A., and Means, A. R. (1991) J. Biol. Chem. 266, 21488–21495
7. George, S. E., Su, Z., Fan, D., and Means, A. R. (1993) J. Biol. Chem. 268, 25213–25220
8. Persechini, A., Ganz, K. J., and Paresi, R. J. (1996) Biochemistry 35, 224–228
9. Persechini, A., McMillan, K., and Leakey, P. (1994) J. Biol. Chem. 269, 16148–16154
10. Nakashima, K., Maekawa, H., and Yazawa, M. (1996) Biochemistry 35, 5602–5610
11. Persechini, A., Paresi, R. J., and Ganss, K. J. (1996) J. Biol. Chem. 271, 19279–19282
12. Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Science 257, 1251–1255
13. Ilera, M., Clore, M., Groenensborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 629–638
14. Newton, D. L., Oldewurtel, M. D., Kirnks, M. H., Shiioh, J., and Klee, C. B. (1984) J. Biol. Chem. 259, 4419–4426
15. Ni, W.-C., and Klee, C. B. (1990) J. Biol. Chem. 265, 6974–6981
16. Stemmer, P. M., and Klee, C. B. (1994) Biochemistry 33, 6859–6866
17. Linse, S., Helmersson, A., and Forsén, S. (1991) J. Biol. Chem. 266, 40550–40554
18. Putkey, J. A., Ono, T., VanBerkum, M. F. A., and Means, A. R. (1988) J. Biol. Chem. 263, 11242–11249
19. VanBerkum, M. F. A., George, S. E., and Means, A. R. (1990) J. Biol. Chem. 265, 3750–3756
20. Persechini, A., and Kretsinger, R. H. (1988) J. Biol. Chem. 263, 12175–12178
21. Persechini, A., Blumenthal, D. K., Jarrett, H. W., Klee, C. B., Hardy, D. O., and Kretsinger, R. H. (1989) J. Biol. Chem. 264, 8052–8058
22. Fitzsimons, D. P., Herring, B. P., Stull, J. T., and Gallagher, P. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11141–11145
23. Herring, B. P. (1991) J. Biol. Chem. 266, 11838–11841
24. George, S. E., VanBerkum, M. F. A., Ono, T., Cook, R., Hanley, R. M., Putkey, J. A., and Means, A. R. (1990) J. Biol. Chem. 265, 9228–9235
25. Su, Z., Blazing, M. A., Fan, D., and George, S. E. (1995) J. Biol. Chem. 270, 29117–29122
26. Shoemaker, M. O., Lau, W., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P.
Localization of Unique Determinants in Calmodulin

E., Guerra-Santos, L., Wilson, E., Lukas, T. J., Van Eldik, L. J., and Watterson, D. M. (1990) *J. Cell. Biol.* **111**, 1107–1125
28. Leachman, S. A., Gallagher, P. J., Herring, B. P., McPhaul, M. J., and Stull, J. T. (1992) *J. Biol. Chem.* **267**, 4930–4938
29. Knighton, D. R., Pearson, R. B., Sowadski, J. M., Means, A. R., Ten Eyk, L. F., Taylor, S. S., and Kemp, B. E. (1992) *Science* **258**, 130–135
30. Herring, B. P., Stull, J. T., and Gallagher, P. J. (1990) *J. Biol. Chem.* **265**, 1724–1730
31. Guerriero, V., Jr., Russo, M. A., Olson, N. J., Putkey, J. A., and Means, A. R. (1986) *Biochemistry* **25**, 8372–8381
32. Goldberg, J., Nairn, A. C., and Kuriyan, J. (1996) *Cell* **84**, 875–887
33. Siivari, K., Zhang, M., Palmer, A. G., III, and Vogel, H. J. (1995) *FEBS Lett.* **366**, 104–108
34. O’Neill, K. T., and DeGrado, W. F. (1990) *Trends Biochem. Sci.* **15**, 59–64
35. Findlay, W. A., Gradwell, M. J., and Bayley, P. M. (1995) *Protein Sci.* **4**, 2375–2382
36. Crouch, T. H., and Klee, C. B. (1980) *Biochemistry* **19**, 3692–3698
37. Maune, J. F., Klee, C. B., and Beckingham, K. (1992) *J. Biol. Chem.* **267**, 5286–5295
38. Forsen, S., Vogel, H. J., and Drakenberg, T. (1986) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. VI, pp. 113–157, Academic Press, New York
39. Mackall, J., and Klee, C. B. (1991) *Biochemistry* **30**, 7242–7247
40. Linse, S., Johansson, C., Brodin, P., Grundstrom, T., Drakenberg, T., and Forsen, S. (1991) *Biochemistry* **30**, 154–162
41. Huang, C. C., Pettersen, E. F., Klein, T. E., Ferrin, T. E., and Langridge, R. (1991) *J. Mol. Graphics* **9**, 239–236
42. Ferrin, T. E., Huang, C. C., Jarvis, L. E., and Langridge, R. (1988) *J. Mol. Graphics* **6**, 13–27
43. Barton, G. J. (1993) *Protein Eng.* **6**, 37–40