Ordered and Cooperative Binding of Opposing Globular Domains of Calmodulin to the Plasma Membrane Ca-ATPase*

Hongye Sun and Thomas C. Squier†

From the Biochemistry and Biophysics Section, Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045-2106

We have investigated the mechanisms of activation of the plasma membrane (PM) Ca-ATPase by calmodulin (CaM), which result in enhanced calcium transport rates and the maintenance of low intracellular calcium levels. We have isolated the amino- or carboxyl-terminal domains of CaM (i.e. CaMN or CaMC), permitting an identification of their relative specificity for binding to sites on either the PM Ca-ATPase or a peptide (C28W) corresponding to the CaM-binding sequence. We find that either CaMN or CaMC alone is capable of productive interactions with the PM Ca-ATPase that induces enzyme activation. There are, however, large differences in the affinity and specificity of binding between CaMN and CaMC and either C28W or the PM Ca-ATPase. The initial binding interaction between CaMN and the PM Ca-ATPase is highly specific, having approximately 10,000-fold greater affinity in comparison with CaMN. However, following the initial association of either CaMC or CaMN, there is a 300-fold enhancement in the affinity of CaMN for the secondary binding site. Thus, while CaMC binds with a high affinity to the two CaM-binding sites within the PM Ca-ATPase in a sequential manner, CaMN binds cooperatively with a lower affinity to both binding sites. These large differences in the binding affinities and specificities of the amino- and carboxyl-terminal domains ensure that CaM binding to the PM Ca-ATPase normally involves the formation of a specific complex in which the initial high affinity association of the carboxyl-terminal domain promotes the association of the amino-terminal domain necessary for enzyme activation.

A range of diverse metabolic activities involved in intracellular signaling is mediated by calmodulin (CaM),1 which functions as the major calcium sensor in all eukaryotes. CaM binding to a range of different target enzymes, including the plasma membrane (PM) Ca-ATPase, has been suggested to result in an increase in enzymatic function as a result of decreased contact interactions between the autoinhibitory domain and catalytic domain elements that result in enhanced rates of substrate binding or utilization (1). CaM binding involves two globular domains, which are connected by an exposed α-helical element often referred to as the central helix (2, 3). Upon calcium binding, the reorientation of α-helices function to expose hydrophobic binding sites within each domain element in CaM that are surrounded by charged amino acids that lead to complex formation and activation of numerous target proteins with little sequence homology (4–6). Backbone folds of the amino- and carboxyl-terminal domains in CaM are structurally similar, and in many instances either domain has been shown to partially activate a range of different target proteins to varying extents (7–9). However, despite the structural homology of the individual CaM binding domains, target protein activation appears to normally involve the specific association of the individual domain elements with specific sequences within the CaM-binding sequence of individual target proteins (7, 9–15). In the case of the PM Ca-ATPase, the carboxyl-terminal domain of CaM has been suggested to be essential for enzyme activation, whereas its amino-terminal domain has been suggested to lack the ability to activate the PM Ca-ATPase without prior association of the carboxyl-terminal domain (16, 17). Thus, differences in the interactions between the amino- and carboxyl-terminal domains of CaM and their corresponding binding sites within target proteins have been suggested to play essential roles in target protein activation. In this respect, previous measurements have demonstrated that the binding preferences of individual CaM-binding domains for sites within the CaM-binding sequence of skeletal myosin light chain kinase are relatively small (i.e. less than 1 kcal/mol), suggesting the possibility of multiple conformations of bound CaM that could function to regulate the extent of target protein activation observed in the presence of saturating CaM concentrations (9, 15). These observations suggest that differences in the maximal extent of enzyme activation for the PM Ca-ATPase and other target proteins observed in the presence of saturating CaM concentrations follow a range of different post-translational modifications (e.g. phosphorylation or methionine oxidation), site-directed deletions, and substitutions of specific amino acids may all involve alterations in the binding mechanism between the opposing globular domains in CaM and target proteins (7, 18–28). It is therefore of interest to identify the binding mechanisms of CaM that normally lead to enzymatic activation of the PM Ca-ATPase. To accomplish this, we have cloned and expressed the amino-terminal domain of CaM and isolated the carboxyl-terminal domain following trypsin digestion and HPLC purification, permitting us to determine the binding specificities of the individual domains of CaM for the CaM-binding sites within the PM Ca-ATPase and the abilities of the individual CaM domains to induce enzyme activation. These measurements take advantage of the fact that the binding affinity and conformation of CaM bound to either a peptide corresponding to the CaM-binding sequence of the PM Ca-

---

* This work was supported by National Institutes of Health Grant AG 12993. 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 correspondence should be addressed. Tel.: 785-864-4008; Fax: 785-864-5321; E-mail: TSQUIER@UKANS.EDU.

1 The abbreviations used are: CaM, calmodulin; CaMN, N-terminal domain of calmodulin containing amino acids 1–77; CaMC, N-terminal domain of calmodulin containing amino acids 78–148; PM, plasma membrane; C28W, a peptide identical to the CaM-binding sequence of the PM Ca-ATPase with the sequence LRGGQLWFGRGLNRIQTQRVTQIVVNAFRISS; HPLC, high performance liquid chromatography.
ATPase activity was measured at 37 °C in a solution containing approx-
imately 0.25 M Trp8 in C28W were assessed through collisional quenching, where
changes associated with CaM binding were detected at 370 nm subse-
quent association with the amino-terminal domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin and soybean trypsin inhibitor (STI) were from Worthington. All other chemicals were obtained from Sigma and were of the purest grade commercially available. The peptide C28W, corre-
ponding to the CaM-binding sequence of the PM Ca-ATPase (LRRG-?
QILWFQRNLRLQYQRHVNAFRSSP), was synthesized and purified by
Quality Control Co. (Hopkinton, MA). The entire vertebrate CaM or a CaM fragment (CaMN) corresponding to first 77 amino acids in CaM
was overexpressed in *Escherichia coli* JM109 (DE3) cells and purified using phenyl-Sepharose CL-4B (Amersham Pharmacia Biotech) chromo-
matography. A fragment corresponding to amino acids 78–148 (CaMC) was obtained following trypsin digestion of CaM. In all cases, CaM or its
individual domain elements were purified using weak anion exchange
HPLC as described previously (30). Erythrocyte ghost membranes con-
taining the PM Ca-ATPase were purified as described previously (21, 31).

**Enzymatic Assays**—The CaM-dependent ATPase activity associated with the PM Ca-ATPase was determined by measuring phosphate re-
lease, essentially as described previously (21, 32). The ghost membrane
protein concentration was determined by the Biuret method (33), using
bovine serum albumin as the standard. CaM concentration was deter-
mined using the Micro-BCA assay (Pierce), where a stock solution of
desalted CaM was used as a protein standard (ε<sub>282</sub> = 3029 M<sup>−1</sup> cm<sup>−1</sup>) (7). ATPase activity was measured at 37 °C in a solution containing approx-
imately 0.25 M trypsin in the presence of variable concentrations of
CaM, CaMN, or CaMC in buffer C (V<sub>c</sub> = 2 ml).

**Determination of CaM Concentrations and Binding Affinities for the PM Ca-ATPase**—The concentration of CaM free in solution was obtained from the following relationship,

$$[\text{CaM}]_{\text{free}} = \frac{(V - V_{\text{max}})}{V_{\text{max}} - V_{\text{min}}} \times [\text{CaM}]_{\text{max}} \quad (\text{Eq. 1})$$

where $V_{\text{max}}$ is the maximal calmodulin-dependent ATPase activity, $V$ is
the observed ATPase activity at a defined concentration of CaM,
$[\text{CaM}]_{\text{total}}$ is the concentration of CaM free in solution, $[\text{CaM}]_{\text{tot}}$ is the total
concentration of CaM added to the solution, and $[\text{CaM}]_{\text{max}}$ is the total
binding capacity of the erythrocyte ghosts for CaM, which was estimated to correspond to 40 pmol of porcine erythrocyte ghost (21).

The CaM-dependent activation of the PM Ca-ATPase by CaM as-
sumes an ordered binding mechanism of CaM with the CaM-binding sites (A-B) of the PM Ca-ATPase (see Scheme I) and is described by the equation,

$$Y = \frac{[\text{PMCA}]_{\text{free}} \times K_1 \times [\text{CaM}]_{\text{free}}}{(1 + K_1 \times [\text{CaM}]_{\text{free}} + K_2 \times [\text{CaM}]_{\text{free}}^2)} \times \text{span + minimum} \quad (\text{Eq. 2})$$

where

$$[\text{PMCA}]_{\text{free}} = -1 + (1 + 4 \times K_2 \times [\text{PMCA}]_{\text{free}}^2 / [\text{PMCA}]_{\text{total}})^{1/2}$$

$$2 \times K_2 \times [\text{CaM}]_{\text{free}}$$

Span is the maximal CaM-dependent enzymatic activity in the presence of
saturating CaM, minimum represents the CaM-independent enzym-
atic activity, and $Y$ represents the PM Ca-ATPase activity resulting from the association of both CaM domains with the Ca-ATPase. The fractional enzyme activity resulting from the association of both CaM domains with the Ca-ATPase. The association constant ($k_1$) for the amino-terminal domain of CaM with the Ca-ATPase. An estimate of the actual
association constant ($k_2$) for the amino-terminal domain can be obtained by taking into account the effective concentration of the amino-terminal domain around the binding site following association of the carboxy-
terminal domain, which equals the following,

$$k_2 = k_3 \times bc$$

The bulk concentration (b) of the amino-terminal domain is assumed to correspond to 1/b<sub>c</sub> (i.e., 10 nm). The concentration of the amino-terminal domain (c) is approximately 1.4 nm and is calculated as follows.

$$c = \frac{1}{N_A \times V} \quad (\text{Eq. 5})$$

$N_A$ is Avogadro’s number, and $V$ is the volume available to the amin-
terminal domain, where the radius corresponds to the overall length of
CaM (approximately 100 Å after association with the carboxy-terminal domain) (36).

The activation of the PM Ca-ATPase by CaMC can be described by the following equation.
Activation Mechanism of the PM Ca-ATPase by Calmodulin

**RESULTS**

**Purification of CaM Fragments**—CaM contains two binding sites that associate with target proteins located in the amino- and carboxyl-terminal domain (1, 6), respectively. Following mild trypsin digestion, it is possible to isolate an amino-terminal fragment containing amino acids Ala1–Lys28 (CaMN') and Asp78–Lys146 (CaMC) using weak anion exchange HPLC. The identity of these fragments was determined using electrospray ionization mass spectrometry; the observed average molecular masses of CaMN' and CaMC are 8316 ± 1 and 8147 ± 1 Da, respectively, in close agreement with the expected monoisotopic molecular mass of 8316.2 and 8147.8 Da for these CaM fragments. These results suggest that previous measurements in which the amino-terminal domain of CaM was reported to be unable to activate the PM Ca-ATPase probably involved CaMN' (16). However, in case Met26 and Lys77 located in the linker region between the amino- and carboxyl-terminal domains might play a role in facilitating target protein binding, we have cloned and expressed the amino-terminal domain of CaM containing amino acids Ala1–Lys77 (CaMN) in E. coli.

CaMN was purified using a phenyl-Sepharose hydrophobic column essentially as described for intact CaM (30), and was subsequently purified using weak anion exchange HPLC. Using electrospray ionization mass spectrometry, we found that CaMN has an observed average molecular mass of 8576 ± 1 Da, in close agreement with the expected average molecular mass of 8575.6 Da.

**CalM-dependent Activation of the PM Ca-ATPase**—CaM activates the PM Ca-ATPase; the amount of CaM necessary for half-maximal activation is 3.9 ± 0.4 nM (Fig. 1). A similar level of enzymatic activation of the PM Ca-ATPase is observed in the presence of saturating concentrations of either CaMN or CaMC. Thus, in contrast to previous reports where CaMN was found to lack the ability to activate the Ca-ATPase (16), we found that the inclusion of two additional amino acids in CaMN provided an amino-terminal domain fully capable of activation of the PM Ca-ATPase. However, the apparent affinities of these isolated domains are dramatically lower; half-maximal activation occurs at 6 ± 1 μM CaMN and 1.7 ± 0.2 μM CaMC (Table I). The 1500- and 400-fold higher concentrations, respectively, of CaMN or CaMC necessary for enzyme activation indicate that (i) individual CaM binding domains are not equivalent and may preferentially interact with one of the two binding sites on the CaM-binding sequence and (ii) that the central helix, which functions to join the individual domains of CaM, facilitates specific association of individual domains with their target sites on the Ca-ATPase. The role of the central helix and possible differences in the binding specificity of individual CaM domains was further assessed by adding an equimolar mixture of CaMN and CaMC, which results in activation of the PM Ca-ATPase, with a half-maximal activation of enzymatic activity occurring with a concentration of both fragments of 0.35 ± 0.02 μM (Fig. 1). The requirement of 100- and 400-fold higher concentrations, respectively, of CaMN or CaMC necessary for enzyme activation relative to that required for intact CaM indicates that the central helix facilitates binding and enzyme activation, in agreement with previous observations (37–39). The approximately 4-fold lower concentration of CaMC necessary for half-maximal activation using a combination of CaMC and CaMN relative to that observed using CaMC alone is consistent with previous suggestions that the carboxyl- and amino-terminal domains of CaM may have different specificities for the two CaM-binding sites.
within the CaM-binding sequence of the PM Ca-ATPase (13). To further understand possible differences in the individual domains of CaM in the activation mechanism of the Ca-ATPase, additional measurements of possible differences in binding specificity are necessary.

**Binding Specificity of Individual Domains to the Plasma Membrane Ca-ATPase—CaM has previously been shown to bind with the same conformation and a comparable affinity to either the PM Ca-ATPase in erythrocyte ghosts or the peptide C28W, corresponding to the CaM-binding sequence of the PM-Ca-ATPase (13, 18, 29, 40). To monitor CaM association with C28W, we have measured the fluorescence emission of Trp8 in C28W, corresponding to the CaM-binding sequence of the PM-Ca-ATPase (13).**

To further understand possible differences in the individual domains of CaM in the activation mechanism of the Ca-ATPase, additional measurements of possible differences in binding specificity are necessary.

**Binding Specificity of Individual Domains to the Plasma Membrane Ca-ATPase—**

CaM has previously been shown to bind with the same conformation and a comparable affinity to either the PM Ca-ATPase in erythrocyte ghosts or the peptide C28W, corresponding to the CaM-binding sequence of the PM-Ca-ATPase (13, 18, 29, 40). To monitor CaM association with C28W, we have measured the fluorescence emission of Trp8 in C28W, corresponding to the CaM-binding sequence of the PM-Ca-ATPase (13). Better understanding possible differences in the individual domains of CaM in the activation mechanism of the Ca-ATPase, additional measurements of possible differences in binding specificity are necessary.

**TABLE I**

*Equilibrium binding affinities between CaM domains and the plasma membrane Ca-ATPase*

| CaM sequence | [CaM](12) | $k_1$ | $k_2$ | $k_3$ |
|--------------|-----------|-------|-------|-------|
| CaM (Ala1–Lys148) | $3.9 \times 10^{-9}$ | $1.0 \times 10^{9}$ | NA | $-1 \times 10^{9}$ |
| CaMN (Ala1–Lys177) | $6 \times 10^{-6}$ | $1 \times 10^{6}$ | NA | $3.2 \times 10^{6}$ |
| CaMC (Asp78–Lys148) | $1.7 \times 10^{-6}$ | $<1.0 \times 10^{6}$ | $0.7 \times 10^{6}$ | NA |
| CaMN + CaMC | $0.4 \times 10^{-6}$ | $<1.0 \times 10^{6}$ | $0.7 \times 10^{6}$ | $2.7 \times 10^{6}$ |

$^a$ NA, not applicable.

$^b$ Estimated affinity based on correction of apparent binding constant ($k_{app} = 5 \times 4 \times 10^9$ M$^{-1}$) for the effective volume available to the amino-terminal domain following association of the carboxyl-terminal domain (see Equation 4).

$^c$ Assumes that CaMN has the same affinity for sites A and B (i.e. $k_c = k_a$ and $k_d = k_b$ in Scheme II).

$^d$ Parameter value based on binding affinity of carboxyl-terminal domain determined for CaM.

$^e$ $k_{b} = 1.1 \times 0.1 \times 10^{9}$ M$^{-1}$ using Equation 7.

$^f$ Parameter value based on activation of PM-Ca-ATPase using CaMC.

$^g$ $k_{b} = 2.8 \times 0.2 \times 10^{9}$ M$^{-1}$ using Equation 7.

---

**Fig. 2. Fluorescence emission spectra of Trp8 in C28W.** Spectra were obtained for $3 \mu M$ C28W, corresponding to the CaM-binding sequence of the PM-Ca-ATPase, in buffer C before (thick solid line; $F_{345} = 1.00$) and after association of $3 \mu M$ CaM (dotted line; $F_{345} = 1.02$), $6 \mu M$ CaMN (dashed line; $F_{345} = 1.18$), or $6 \mu M$ CaMC (thin solid line; $F_{345} = 0.92$). Excitation was $297$ nm.
The CaM-dependent activation of the PM Ca-ATPase to Equation 2, which assumes an ordered binding mechanism of CaM with the Ca-ATPase (see Scheme I). A least squares fit to the data for PM Ca-ATPase activation by intact CaM in Fig. 1 indicates that \( k_1 = 1.0 \pm 0.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \) and \( k_{20} = 5 \pm 4 \times 10^9 \text{ M}^{-1} \). Following correction for the large increase in the effective concentration of the amino-terminal domain following binding of the carboxyl-terminal domain (see Equation 4), we can estimate that the equilibrium binding constant for the amino-terminal domain for site B within the CaM-binding sequence of the PM Ca-ATPase is approximately \( 1 \times 10^6 \text{ M}^{-1} \) (Table I), in agreement with previous determinations of the affinity of the amino-terminal domain of CaM to phosphodiesterase, myosin light chain kinase, and nitric-oxide synthase (7–9, 15).

In the case of a homogeneous population of a single domain of CaM (i.e. CaMN or CaMC), the activation of the PM Ca-ATPase (7) requires that the individual domains of CaM associate with both binding sites within the CaM-binding sequence of the PM Ca-ATPase. This is equivalent to a single ligand binding to multiple sites on an enzyme, where occupancy of both sites is necessary for activation, and can be described using Equation 6 for the activation of the PM Ca-ATPase by CaMC. Fitting the data in Fig. 1 for CaMC, we are unable to obtain an accurate estimate of \( k_1 \). However, if one assumes that \( k_1 \) equals \( 1.0 \times 10^{9} \text{ M}^{-1} \) as determined for intact CaM (see above), then a least squares fit to the data indicates that \( k_2 \) equals \( 0.7 \pm 0.1 \times 10^6 \text{ M}^{-1} \) (Table I). Since \( k_1 \gg k_2 \), site A is essentially completely filled prior to the titration of site B (see Scheme I), which is associated with the activation of the Ca-ATPase. Under these latter conditions, the binding affinity of site B can be described by the simple binding equation (see Equation 7), which adequately describes the data in Fig. 1. A least squares fit to the data indicates that \( k_2 \) equals \( 1.1 \pm 0.1 \times 10^6 \text{ M}^{-1} \). The latter estimate of the binding affinity is in close agreement to that obtained using Equation 6 and is consistent with the CaMC concentration necessary for half-maximal maximal activation (i.e. \([\text{CaMC}]_{0.5} = 1.7 \pm 0.2 \mu\text{M}\). The activation of the PM Ca-ATPase by CaMN requires that binding to sites A and B on C28W in Scheme II be taken into account that CaM contains two binding domains and that both domains must associate with binding sites within the CaM-binding sequence of the PM Ca-ATPase to induce enzyme activation, in analogy to other CaM-dependent enzymes (9, 15).

The CaM-dependent activation of the PM Ca-ATPase by CaM, CaMN, or CaMC was used to estimate the binding affinities between individual domains of CaM and the CaM-binding sequence of PM Ca-ATPase. This analysis takes into account that CaM contains two binding domains and that both domains must associate with binding sites within the CaM-binding sequence of the PM Ca-ATPase to induce enzyme activation, in analogy to other CaM-dependent enzymes (9, 15). Initial binding between CaM and target proteins, including the PM Ca-ATPase, has been suggested to involve the high affinity association of the carboxyl-terminal domain of CaM (Refs. 1 and 16; see above), and that association of the amino-terminal domain is aided by the reduced volume available for its diffusion due to the association of the carboxyl-terminal domain of CaM to the PM Ca-ATPase (9, 37, 38). Therefore, we have fit the CaM-dependent activation of the PM Ca-ATPase to Equation 2, which assumes an ordered binding mechanism of CaM with the Ca-ATPase (see Scheme I). A least squares fit to the data for PM Ca-ATPase activation by intact CaM in Fig. 1 indicates that \( k_1 = 1.0 \pm 0.1 \times 10^9 \text{ M}^{-1} \) and \( k_{20} = 5 \pm 4 \times 10^9 \text{ M}^{-1} \). Following correction for the large increase in the effective concentration of the amino-terminal domain following binding of the carboxyl-terminal domain (see Equation 4), we can estimate that the equilibrium binding constant for the amino-terminal domain for site B within the CaM-binding sequence of the PM Ca-ATPase is approximately \( 1 \times 10^6 \text{ M}^{-1} \) (Table I), in agreement with previous determinations of the affinity of the amino-terminal domain of CaM to phosphodiesterase, myosin light chain kinase, and nitric-oxide synthase (7–9, 15).

In the case of a homogeneous population of a single domain of CaM (i.e. CaMN or CaMC), the activation of the PM Ca-ATPase (7) requires that the individual domains of CaM associate with both binding sites within the CaM-binding sequence of the PM Ca-ATPase. This is equivalent to a single ligand binding to multiple sites on an enzyme, where occupancy of both sites is necessary for activation, and can be described using Equation 6 for the activation of the PM Ca-ATPase by CaMC. Fitting the data in Fig. 1 for CaMC, we are unable to obtain an accurate estimate of \( k_1 \). However, if one assumes that \( k_1 \) equals \( 1.0 \times 10^9 \text{ M}^{-1} \) as determined for intact CaM (see above), then a least squares fit to the data indicates that \( k_2 \) equals \( 0.7 \pm 0.1 \times 10^6 \text{ M}^{-1} \) (Table I). Since \( k_1 \gg k_2 \), site A is essentially completely filled prior to the titration of site B (see Scheme I), which is associated with the activation of the Ca-ATPase. Under these latter conditions, the binding affinity of site B can be described by the simple binding equation (see Equation 7), which adequately describes the data in Fig. 1. A least squares fit to the data indicates that \( k_2 \) equals \( 1.1 \pm 0.1 \times 10^6 \text{ M}^{-1} \). The latter estimate of the binding affinity is in close agreement to that obtained using Equation 6 and is consistent with the CaMC concentration necessary for half-maximal maximal activation (i.e. \([\text{CaMC}]_{0.5} = 1.7 \pm 0.2 \mu\text{M}\). The activation of the PM Ca-ATPase by CaMN requires that binding to sites A and B on C28W in Scheme II be taken into account that CaM contains two binding domains and that both domains must associate with binding sites within the CaM-binding sequence of the PM Ca-ATPase to induce enzyme activation, in analogy to other CaM-dependent enzymes (9, 15).
Activation Mechanism of the PM Ca-ATPase by Calmodulin

account, since the data in Fig. 1 cannot be fit to a simple model involving a homogeneous population of binding sites as observed for CaMC (see above). Therefore, we have fit the CaMN-dependent activation of the PM Ca-ATPase using Equation 8. If one assumes that $k_1 = k_2$ and $k_3 = k_4$, then $k_1$ equals $1 \pm 1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and $k_3$ equals $3.2 \pm 0.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Table I). These values indicate that occupancy of one binding site with CaMN induces cooperative structural changes that enhance the affinity of CaMN binding to the other site. Similar binding affinities were obtained for CaMN binding to gizzard myosin light chain kinase (9), suggesting that the low affinity and cooperative association of the amino-terminal domain with some target enzymes may be a general feature involving CaM binding that is fundamental to their activation mechanism.

Additional understanding of the binding mechanism of CaM to the PM Ca-ATPase is possible from a consideration of the activation of the Ca-ATPase by a mixture of CaM domains, which is simplified by the substantially larger binding affinity and specificity of the carboxyl-terminal domain of CaM for site A within the CaM-binding sequences of the Ca-ATPase relative to the amino-terminal domain (see above). Therefore, upon titration of the PM Ca-ATPase with an equal concentration of the amino- and carboxyl-terminal domains, the high affinity association of the carboxyl-terminal domain ensures that enzyme activation (Y) is the result of the association of either the amino- or carboxyl-terminal domain with a single site (i.e. site B in Scheme I) on the PM Ca-ATPase, which can be described using Equation 9. Consistent with the binding affinity determined from a consideration of the CaMN-dependent activation of the PM Ca-ATPase, a least squares fit to the data in Fig. 1 indicates that for a mixture of CaMN and CaMC that $k_3$ equals $2.7 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, where $k_1$ and $k_3$ are independently determined from a consideration of the activation of the PM Ca-ATPase by either CaM or CaMC, respectively. Thus, occupancy of site A enhances that affinity of CaMN by approximately 300-fold, consistent with earlier results obtained using gizzard myosin light chain kinase (9). These results indicate that a cooperative binding mechanism between the carboxyl- and amino-terminal domains of CaM through structural changes involving the CaM-binding sequence is important in facilitating CaM binding and that the central helix is not necessary to promote cooperative binding and enzyme activation.

**DISCUSSION**

The isolated amino- and carboxyl-terminal domains of CaM (i.e. CaMN and CaMC) are both able to fully activate the PM Ca-ATPase (Fig. 1), indicating that these structurally homologous domains are able to bind productively to either binding site within the CaM-binding sequence of the PM Ca-ATPase so as to induce the normal structural changes associated with enzyme activation. However, while CaMC binds sequentially to the two sites within the CaM-binding sequence with high affinity, we find that CaMN binds with a lower affinity to both sites in a highly cooperative manner (Figs. 3 and 4; Table I). These large differences with respect to the affinity and binding specificity are the result of a 10,000-fold higher affinity for the initial binding interaction between CaMC and the CaM-binding sequence of the PM Ca-ATPase in comparison with that observed for CaMN, which functions to ensure a unique orientation of the complex between CaM and the CaM-binding sequence of the PM Ca-ATPase necessary for productive binding and enzyme activation. The two binding sites within the CaM-binding sequence of the PM Ca-ATPase are structurally coupled, as indicated by the biphasic fluorescence changes and the cooperative binding between CaMN domains that results in a 300-fold increase in the binding affinity for the secondary site as a result of the occupancy of the primary binding site (Fig. 3; Table I). However, following the initial association of either domain, the affinity of CaMC or CaMN for the secondary site is very similar (Table I). These results suggest that the molecular determinants that define binding to the secondary site are relatively nonspecific and emphasize that the binding specificity of the carboxyl-terminal domain defines the correct orientation of the complex between CaM and the CaM-binding sequence of the PM Ca-ATPase. Thus, the initial specific binding of CaMC to the high affinity site promotes the cooperative binding of CaMN to the secondary site necessary for rapid enzyme activation.

**Role of Individual CaM Domains in Promoting Enzyme Activation**—Previous measurements demonstrated that an HPLC-purified carboxyl-terminal fragment following trypsin digestion (CaMC) is able to fully activate the PM Ca-ATPase (16), in agreement with the present results (see Fig. 1). In contrast, the isolated amino-terminal fragment purified following trypsin digestion of CaM (now known to correspond to CaMN′) was unable to significantly activate the PM Ca-ATPase or a range of other CaM-dependent enzymes in the absence of CaMC (7, 9, 16), suggesting that there may be distinct structural requirements associated with the recognition elements of each CaM domain and the corresponding binding sites on the PM Ca-ATPase. In contrast, we have demonstrated using CaMC and CaMN that either domain of CaM can fully activate the PM Ca-ATPase (Fig. 1). Differences between our results and those previously reported may be related to the additional two amino acids (i.e. Met76 and Lys77) found in CaMN relative to CaMN′. Thus, the inability of CaMN′ to activate the PM Ca-ATPase may be the result of a requirement for specific binding interactions involving Met76 or Lys77 either with the CaM-binding sequence of the PM Ca-ATPase or other residues within the amino-terminal domain of CaM that stabilizes the tertiary structure of CaMN. However, irrespective of the physical reasons, these results indicate that either domain of CaM is able to productively associate with the CaM-binding sequence of the PM Ca-ATPase to promote enzyme activation, and they emphasize the flexibility of the recognition elements within individual CaM binding domains that promotes binding to a range of different sequences. Thus, under equilibrium conditions, the PM Ca-ATPase has minimal structural requirements for specific side chain interactions involving either CaM domain to promote enzyme activation of the PM Ca-ATPase, and CaM-dependent activation appears to be simply a matter of occupying both binding sites to promote enzyme activation on a time scale appropriate to calcium signaling. However, because of the large differences in the binding affinities of the amino- and carboxyl-terminal domains (Table I), under normal physiological conditions CaM binds to the CaM-binding sequence of the PM Ca-ATPase in a unique orientation, with the carboxyl-terminal domain of CaM in association with the amino-terminal portion of the CaM-binding peptide. The latter binding orientation is in agreement with previous measurements obtained through a consideration of intermolecular distances between CaM and C28W obtained using fluorescence resonance energy transfer (13).

**Proposed Binding Mechanism of CaM to the PM Ca-ATPase**—The specific binding of CaM to the PM Ca-ATPase in a unique orientation is the result of the 10,000-fold higher affinity of the carboxyl-terminal domain for the primary binding site (i.e. site A in Scheme I) relative to the amino-terminal domain. Following initial association of the carboxyl-terminal domain with the CaM-binding site of the PM Ca-ATPase, cooperative structural changes within the CaM-binding sequence enhance the binding of the amino-terminal domain necessary for enzyme activation. In addition, there is a 40,000-fold in-
crease in the effective concentration of the amino-terminal domain following the binding of the carboxyl-terminal domain as a result of the structural linkage between these domains through the central helix (see Equation 4). These results suggest that there is no need for a high intrinsic affinity between the amino-terminal domain and the CaM-binding sequence in order to quickly saturate the secondary binding site in the presence of activating calcium. Similar cooperative binding interactions have been observed between individual CaM domains and the CaM-binding sequence in myosin light chain kinase (9), suggesting that enhanced binding interactions involving a structural linkage between two binding sites of the CaM-binding sequence may be a common feature for CaM-dependent enzymes. Since the crystal structures of CaM bound to the CaM-binding sequences of myosin light chain kinase or multifunctional CaM-dependent kinase II indicate that the CaM-binding sequences adopt a-helical structure, the basis for these cooperative interactions may involve secondary structural changes within the CaM-binding sequence following association of the carboxyl-terminal domain. It is interesting that the binding constant of the amino-terminal domain in intact CaM is approximately 1 order of magnitude weaker than that of CaMN (Table I), suggesting that steric constraints imposed by the central helix restrict the binding interaction. However, from a practical point of view, the 40,000-fold enhancement in the apparent binding affinity (k_{app}) compensates for the small reduction in k_d for the amino-terminal domain in intact CaM relative to that of CaMN and is sufficient to ensure the saturation of the secondary binding site by the amino-terminal domain under conditions of activating calcium.

Possible Physiological Role for the Differential Binding of Individual CaM Domains—Large differences in the binding affinities of the two domains in CaM have previously been suggested to offer important advantages in terms of rapidly regulating enzyme function (14, 36). It is therefore possible for the high affinity carboxyl-terminal domain to preferentially bind to target enzymes at low calcium concentrations so as to promote the rapid binding of the amino-terminal domain necessary for enzyme activation following transient increases in cytosolic calcium concentrations. In addition, post-translational modifications that alter the apparent affinity of the amino-terminal domain can differentially activate target protein function by modifying the binding of the amino-terminal domain necessary for enzyme activation (9, 14). Thus, enzymes such as neuronal nitric-oxide synthase have been suggested to bind CaM at low calcium concentrations through the modulation of the affinity of the amino terminus and the CaM-binding sequence (9). Likewise, the oxidative modification of methionines in CaM have been shown to preferentially decrease the binding affinity of the amino terminus of CaM to the CaM-binding sequence of the PM Ca-ATPase and to decrease the maximal activation in the presence of saturating CaM concentrations (21, 27, 35). It should be noted that a large difference in binding affinity is required to permit the selective binding of the carboxyl-terminal domain of CaM at low (i.e. resting) calcium levels, since the presence of target peptides has previously been shown to result in a 100-fold increase in the apparent calcium affinity of CaM (41). Therefore, the approximately 100-fold difference in the affinities of the carboxyl-terminal domain for site A and the amino-terminal domain for site B following association of the carboxyl-terminal domain suggest that at low calcium concentrations the carboxyl-terminal domain (which has a 10-fold higher calcium affinity) (42) may preferentially associate with the PM Ca-ATPase in an inactive conformation. Under these conditions, the enhanced calcium affinity of the individual domains of CaM in the presence of target peptides would not be enough to promote binding of the amino-terminal domain. Small increases in calcium concentrations would result in rapid binding of the amino-terminal domain, which has the potential to ensure the rapid activation of the PM Ca-ATPase necessary for rapid calcium resequestration. Likewise, small decreases in the binding affinity as a result of post-translational modifications would have the potential to result in a reduction in the maximal levels of activation, consistent with the observation that oxidized CaM functions as an antagonist with respect to the activation of the PM Ca-ATPase by native (unoxidized) CaM (21).

Conclusions and Future Directions—We have demonstrated that occupancy of the two binding sites within the CaM-binding sequence of the PM Ca-ATPase with either domain of CaM is sufficient to fully activate the PM Ca-ATPase, indicating that there are no specific requirements involving side chain interactions between individual CaM-binding domains and the PM Ca-ATPase necessary for enzyme activation. However, under normal conditions, the carboxyl-terminal domain specifically associates with a unique site near the amino terminus of the CaM-binding sequence. Following association of the carboxyl-terminal domain, cooperative structural changes between the primary and secondary binding sites within the CaM-binding sequence and the large increase in the effective concentration of the amino-terminal domain function to enhance the binding of the amino-terminal domain necessary for enzyme activation. Future studies need to correlate structural changes within the CaM-binding sequence to occupancy of the individual binding sites and investigate the influence of post-translational modifications involving both CaM and the PM Ca-ATPase on the binding affinities and cooperative interactions between individual CaM domains and binding sites on the PM Ca-ATPase.

Acknowledgments—We thank Diana J. Bigelow for insightful discussions and Dan Yin and Robert F. Weaver for advice and technical assistance.

REFERENCES
1. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 85–116
2. Burke, Y. S., Bugg, C. E., and Cook, W. J. A. (1988) J. Mol. Biol. 204, 191–204
3. Chattopadhyaya, R., Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) J. Mol. Biol. 228, 1177–1192
4. Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C. B., and Bax, A. (1995) Nat. Struct. Biol. 2, 768–776
5. Zhang, M., Tanaka, T., and Ikura, M. (1995) Nat. Struct. Biol. 2, 758–767
6. Tjandra, N., Bax, A., Crivici, A., and Ikura, M. (1995) in Calcium in Cellular Regulation (Carafoli, E., and Klee, C., eds) pp. 152–170, Oxford University Press, Oxford
7. Newton, D. L., Oldewurtel, M. D., Kinka, M. H., Shiloach, J., and Klee, C. B. (1984) J. Biol. Chem. 259, 4419–4426
8. Newton, D. L., Klee, C., Woodgett, J., and Cohen, P. (1985) Biochem. Biophys. Acta 845, 533–539
9. Persechini, A., McMillan, K., and Leakey, P. (1994) J. Biol. Chem. 269, 10473–10474
10. Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Science 257, 1251–1254
11. Meador, W. E., Means, A. R., and Quiocho, F. A. (1993) Science 262, 1718–1721
12. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 653–658
13. Chapman, E. R., Alexander, K., Vorherr, T., Carafoli, E., and Storm, D. R. (1992) Biochemistry 31, 12819–12825
14. Bayley, P. M., Findlay, W. A., and Martin, S. R. (1996) Protein Sci. 5, 1215–1228
15. Bartb, A., Martin, S. R., and Bayley, P. M. (1998) J. Biol. Chem. 273, 2174–2183
16. Guerini, D., Krebs, J., and Carafoli, E. (1984) J. Biol. Chem. 259, 15172–15177
17. Elshor, B., Hennig, M., Försterling, H., Diener, A., Maurer, M., Schlute, P., Chwalbe, H., Friesinger, C., Krebs, J., Schindl, H., Vorherr, T., and Carafoli, E. (1990) Biochemistry 29, 12330–12332
18. Vorherr, T., James, P., Krebs, J., Eyned, A., McCormick, D. J., Penniston, J. T., and Carafoli, E. (1990) Biochemistry 29, 355–365
19. Quadroni, M., L’Hostis, E. L., Corti, C., Myagkikh, I., Durusseau, I., Cox, J., James, P., and Carafoli, E. (1998) Biochemistry 37, 6523–6532
20. Zhang, M., Li, M., Wang, J. H., and Vogel, H. J. (1994) J. Biol. Chem. 269, 15546–15552
21. Yao, Y., Yin, D., Jas, G., Kuczerka, K., Williams, D. T., Schöneich, C., and Squier, T. C. (1996) Biochemistry 35, 2767–2778
22. Perschini, A., Ganz, K. J., and Pareis, R. J. (1996) J. Biol. Chem. 271, 19279–19282
23. Chin, D., and Means, A. R. (1996) *J. Biol. Chem.* **271**, 30465–30471
24. Chin, D., Soan, D. J., Quiocho, F. A., and Means, A. R. (1997) *J. Biol. Chem.* **272**, 3510–3513
25. Chin, D., Winkler, K. E., and Means, A. R. (1997) *J. Biol. Chem.* **272**, 31235–31240
26. Edwards, R. A., Walsh, M. P., Sutherland, C., and Vogel, H. J. (1998) *Biochem. J.* **331**, 149–152
27. Gao, J., Yin, D., Yao, Y., Williams, T. D., and Squier, T. C. (1998) *Biochemistry* **37**, 9536–9548
28. Yuan, T., and Vogel, H. J. (1999) *Protein Sci.* **8**, 113–121
29. Yao, Y., Gao, J., and Squier, T. C. (1996) *Biochemistry* **35**, 12015–12028
30. Sun, H., Yin, D., and Squier, T. C. (1999) *Biochemistry* **38**, 12266–12279
31. Niggli, V., Penniston, J. T., and Carafoli, E. (1979) *J. Biol. Chem.* **254**, 9955–9958
32. Lanzetta, P. A., Alvarez, L. J., Reinsch, P. S., and Candia, O. (1979) *Anal. Biochem.* **100**, 95–97
33. Gornal, A., Bardawill, C., and David, M. (1949) *J. Biol. Chem.* **177**, 751–766
34. Fabiato, A. (1988) *Methods Enzymol.* **157**, 378–417
35. Gao, J., Yin, D. H., Yao, Y., Sun, H., Qin, Z., Schöneich, C., Williams, T. D., and Squier, T. C. (1998) *Biophys. J.* **74**, 1115–1134
36. Kruegler, J. K., Bishop, N. A., Blumenthal, D. K., Zhi, G., Beckingham, K., Stull, J. T., and Trehwellia, J. (1998) *Biochemistry* **37**, 17810–17817
37. Persechini, A., and Kretsinger, R. H. (1988) *J. Biol. Chem.* **263**, 12175–12178
38. Persechini, A., Jarrett, H. W., Kesk-Kosikka, D., Krinks, M. H., and Lee, H. G. (1993) *Biochim. Biophys. Acta* **1163**, 309–314
39. Persechini, A., Ganez, K. J., and Paresi, R. J. (1996) *Biochemistry* **35**, 224–228
40. Yao, Y., and Squier, T. C. (1996) *Biochemistry* **35**, 6815–6827
41. Peersen, O. B., Madsen, T. S., and Falke, J. J. (1997) *Protein Sci.* **6**, 794–807
42. Klee, C. B. (1988) in *Calmodulin* (Cohen, P., and Klee, C. B., eds) pp. 35–46, Elsevier Science Publishers B.V., Amsterdam