Nonessential Role for Methionines in the Productive Association between Calmodulin and the Plasma Membrane Ca-ATPase†

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ABSTRACT: To investigate the role of hydrophobic interactions involving methionine side chains in facilitating the productive association between calmodulin (CaM) and the plasma membrane (PM) Ca-ATPase, we have substituted the polar amino acid Gln for Met at multiple positions in both the amino- and carboxyl-terminal domains of CaM. Conformationally sensitive fluorescence signals indicate that these mutations have little effect on the backbone fold of the carboxyl-terminal domain of CaM. The insertion of multiple Gln in either globular domain results in a decrease in the apparent affinity of CaM for the PM-Ca-ATPase. However, despite the multiple substitution of Gln for four methionines at positions 36, 51, 71, and 72 in the amino-terminal domain or for three methionines at positions 124, 144, and 145 in the carboxyl-terminal domain, these mutant CaMs are able to fully activate the PM-Ca-ATPase. Thus, although these CaM mutants have a decreased affinity for the CaM-binding site on the Ca-ATPase, they retain the ability to fully activate the Ca-ATPase at saturating concentrations of CaM. The role of individual methionines in modulating the affinity between the carboxyl terminus and the PM-Ca-ATPase was further investigated through the substitution of individual Met with Gln. Upon substitution of Met^{124} and Met^{144} with Gln, there is a 5- and 10-fold increase in the amount of CaM necessary to obtain half-maximal activation of the PM-Ca-ATPase, indicating that these methionine side chains participate in the high-affinity association between CaM and the PM-Ca-ATPase. However, substitution of Gln for Met^{145} results in no change in the apparent affinity between CaM and the PM-Ca-ATPase, indicating that in contrast to all other known CaM targets, Met^{145} does not participate in the interaction between CaM and the PM-Ca-ATPase. These results emphasize differences in the binding interactions between individual methionines in CaM and different target enzymes, and suggest that hydrophobic interactions between methionines in CaM and the binding site on the PM-Ca-ATPase are not necessary for enzyme activation. Calculation of the binding affinities of individual CaM domains associated with activation of the PM-Ca-ATPase suggests that mutations of methionines located in either domain of CaM can decrease the initial high-affinity association between CaM and the PM-Ca-ATPase, but have little effect upon the subsequent binding of the opposing globular domain. These results suggest that the initial associations between CaM and the CaM-binding sequence in the PM-Ca-ATPase are guided by nonspecific hydrophobic interactions involving both domains of CaM.

Calmodulin (CaM)[1] functions to recognize the calcium signal in all eukaryotic cells, and is involved in the coordinate regulation of diverse target proteins involved in modulating a range of biological responses, including neurotransmission, muscle contraction, glucose metabolism, cell proliferation, and gene expression (*1*, *2*). Upon calcium activation, hydrophobic binding surfaces are exposed on each of the opposing globular domains (*3*, *4*). Methionine side chains contribute approximately one-half of the exposed hydrophobic binding surface on each of the opposing globular domains, and the nonpolar thioether moiety has been suggested to provide the necessary conformational flexibility to permit the high-affinity association between CaM and a range of different target proteins with little sequence homology (*5*−*8*). Thus, essentially all methionine side chains make van der Waals contact interactions within the three high-resolution structures of CaM bound to peptides identical to the CaM-binding sequences of CaM-dependent protein kinase IIα, smooth muscle myosin light chain kinase (MLCK), or skeletal MLCK (*9*−*11*). However, the relative contribution of individual methionines in stabilizing the bound conformation is highly variable, and amino acid substitutions involving the insertion of glutamine for individual methionines differentially alter the binding and activation of these three different target enzymes by CaM (*12*). Furthermore, different

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[1] Abbreviations: CaM, calmodulin; CaMKI, calmodulin-dependent protein kinase I; CaMKII, calmodulin-dependent protein kinase II; CaMKIV, calmodulin-dependent protein kinase IV; CaM_{ox}, oxidatively modified CaM; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; MLCK, myosin light chain kinase; PCR, polymerase chain reaction; PM, plasma membrane.

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hydrophobic interactions between CaM and various target proteins may be involved in the binding and subsequent activation of target proteins (*13*, *14*). In this respect, posttranslational modifications involving the oxidative modification of selected methionines within CaM (i.e., CaM_{ox}) occur during normal biological aging, resulting in an altered structural interaction between CaM_{ox} and the Ca-ATPase that does not fully activate the PM-Ca-ATPase when bound to CaM_{ox} (*15*−*18*). Since substitutions of Gln for specific Met have been shown to reduce the ability of CaM to fully activate MLCK and CaM-dependent protein kinase (*12*), these results suggest that the decrease in the ability of CaM_{ox} to fully activate the PM-Ca-ATPase could be the result of either (i) the loss of specific binding interactions involving methionine side chains necessary to induce enzyme activation or (ii) differences in a range of binding interactions that result from global conformational changes in CaM_{ox}. To explore the possible role of alterations in specific hydrophobic binding interactions between individual methionines in CaM in the activation of the PM-Ca-ATPase, we have used site-directed mutagenesis to generate CaM mutants in which variable numbers of methionines in both the amino- and carboxyl-terminal domains of CaM were substituted with polar glutamine. An examination of the CaM-dependent activation of the PM-Ca-ATPase using these mutant CaMs reveals that although the majority of methionine mutations (with the exception of $Met^{145} \rightarrow Gln^{145}$) affect the binding affinity between CaM and the PM-Ca-ATPase, that the substitution of multiple glutamines for methionines in either domain of CaM does not affect the ability of CaM to fully activate the PM-Ca-ATPase at saturating CaM concentrations. Thus, we find that (i) methionine side chains are not critical to the ability of CaM to fully activate the PM-Ca-ATPase at saturating CaM concentrations and (ii) Met^{145} is not involved in binding to the PM-Ca-ATPase. These latter results are in contrast to CaM-dependent protein kinase IIα or smooth muscle MLCK, where significant changes in maximal enzyme activation were observed following substitution of some methionines with glutamines (*12*, *14*). These results indicate that CaM binds differently to the PM-Ca-ATPase, suggesting that interactions between methionine side chains and sequences within the CaM-binding sequence of the Ca-ATPase are not essential for complete enzymatic activation.

EXPERIMENTAL PROCEDURES

Materials. *Pfu* DNA polymerase, restriction enzymes *Nco*I and *Xba*I, and DNA endonuclease *Dpn*I were obtained from Stratagene (La Jolla, CA). Complementary oligonucleotides used for site-directed mutagenesis were obtained from Genosys (Woodlands, TX). Phenyl-Sepharose CL-4B was obtained from Pharmacia (Piscataway, NJ). HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] and TRIS free base [tris(hydroxymethyl)aminomethane] were purchased from Research Organics Inc. (Cleveland, OH). A Micro-BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). All other chemicals were the purest grade commercially available. The cDNA encoding chicken CaM, provided by Professor Sam George (Duke University), was subcloned into the expression vector pALTER-Ex1 (Promega, Madison, WI) downstream form a T7 promoter, overexpressed in *E. coli* strain JM109(DE3) (Promega), and purified essentially as previously described using phenyl-Sepharose CL-4B and weak anion exchange HPLC (*19*, *20*). The erythrocyte ghost PM-Ca-ATPase was purified from porcine erythrocyte ghost membranes (*21*). Purified CaM and erythrocyte ghost membranes were stored at −70 °C.

Construction of CaM Mutants. The recombinant plasmid pEx1-CaM was incubated with a pair of complementary oligonucleotides in which the appropriate ATG codons for Met were changed to CAG for Gln, and through 16 rounds of the polymerase chain reaction (PCR), individual methionine codons were substituted with glutamine codons. Original DNA templates were digested with *Dpn*I prior to the transformation of *E. coli* JM109(DE3) cells with the PCR product. Plasmids were purified from the transformed cells and subjected to automated DNA sequencing using the Biochemical Research Service Laboratory at the University of Kansas. The mass of the expressed mutant CaM was obtained using ESI mass spectrometry, as previously described (*17*, *20*).

Enzymatic Assays. The calmodulin-dependent ATPase activity associated with the PM-Ca-ATPase was determined using the method described by Lanzetta and co-workers (*22*) for measuring phosphate release. The ghost membrane protein concentration was determined by the Biuret method (*23*), using BSA as the standard. CaM concentration was determined using the Micro-BCA assay, where a stock solution of desalted CaM was used as a protein standard ($\epsilon_{277} = 3029\ M^{-1}\ cm^{-1}$; *19*). ATPase activity was measured at 37 °C in a solution containing approximately 16 nM Ca-ATPase (i.e., 0.4 mg mL^{-1} porcine erythrocyte ghost membranes) in 100 mM HEPES (pH 7.5), 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.44 mM $CaCl_2$, 5 mM ATP, and 4 μM A23187. The free calcium concentration was calculated to be 100 μM (*24*).

Calculation of Free CaM Concentrations. The concentration of free CaM was obtained from the following relationship:

$$[CaM]_{free} = [CaM]_{total} - \frac{(V - V_{min})}{(V_{max} - V_{min})} \times [PM\text{-}Ca\text{-}ATPase] \quad (1)$$

where V_{max} is the maximal calmodulin-dependent ATPase activity, V is the observed ATPase activity at a defined concentration of CaM, $[CaM]_{free}$ is the concentration of CaM free in solution, $[CaM]_{total}$ is the total concentration of CaM added to the solution, and [PM-Ca-ATPase] is the total binding capacity of the erythrocyte ghosts for CaM, which was measured to be 40 pmol of CaM bound/mg of porcine erythrocyte ghost (*25*).

Fluorescence Spectroscopy Measurements. Steady-state fluorescence intensities were measured using a Fluoro Max-2 (Jobin Yvon Spex, Edison, NJ) equipped with a xenon lamp. Excitation was at 297 nm using 4 nm slit-widths on both the excitation and emission monochromators.

RESULTS

The PM-Ca-ATPase contains an autoinhibitory domain near the carboxyl terminus that functions to block substrate access or utilization (*25*, *26*). Upon binding CaM, there is a

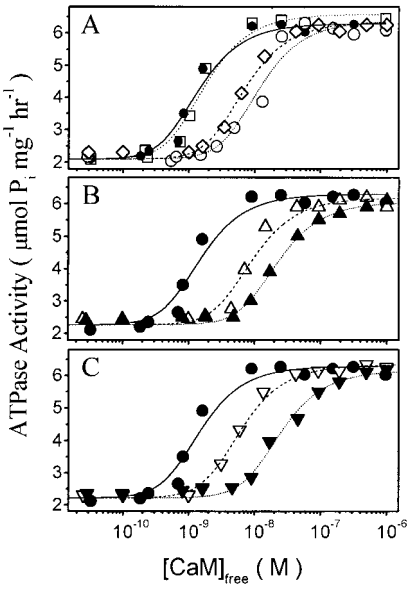

FIGURE 1: Calmodulin-dependent activation of the PM-Ca-ATPase. ATPase activity for wild-type CaM (●; solid line) is compared with mutant CaMs (Y99W) involving: (A) single methionine substitutions in the C-terminus involving M124Q (◇; dashed line), M144Q (○; short dashed line), and M145Q (□; dotted line); (B) multiple methionine substitutions in the carboxyl-terminal domain involving the substitution of Gln for Met^{144} and Met^{145} (i.e., C-2Q; △; dashed line) or Met^{124}, Met^{144}, and Met^{145} (i.e., C-3Q; ▲; dotted line); and (C) multiple methionine substitutions in the amino-terminal domain involving the substitution of Gln for Met^{71} and Met^{72} (N-2Q; ▽; dashed line) or Met^{36}, Met^{51}, Met^{71}, and Met^{72} (i.e., N-4Q; ▼; dotted line). Lines represent the least-squares fit to the data, as previously described (*30*). ATPase activity was measured in the presence of 0.4 mg mL^{-1} porcine erythrocyte ghost membranes (i.e., 16 nM PM-Ca-ATPase; *25*) in 0.1 M HEPES (pH 7.5), 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.44 mM $CaCl_2$, 5 mM ATP, and 6 μM A23187 at 37 °C, where the concentration of unbound CaM was calculated using eq 1 under Experimental Procedures. The free calcium concentration was calculated to be 100 μM (*24*).

3-fold activation in the catalytic activity of the PM-Ca-ATPase (Figure 1). The binding mechanism between CaM and a range of different target proteins, including the PM-Ca-ATPase, is thought to involve hydrophobic binding interactions that stabilize the CaM-binding sequence of the target protein in a conformation that results in enzymatic activation (*8*). Methionines within the binding clefts of CaM represent approximately 46% of the binding surface, and are critical to the maximal activation of myosin light chain kinase and CaM-dependent protein kinase IIα (*6*, *9*−*11*). To define the role of these hydrophobic interactions in the productive association between CaM and the PM-Ca-ATPase, we have used site-directed mutagenesis to substitute individual methionines with glutamines. The greater polarity of the glutamine side chain relative to methionine is expected to decrease hydrophobic interactions that would normally stabilize the binding interaction between CaM and the CaM-binding sequence of the PM-Ca-ATPase that may be necessary for enzyme activation. Because Gln substitutions are not expected to alter the α-helical secondary structure (*27*), these mutations permit an assessment of the influence of specific methionine side-chain interactions in the association with the CaM-binding sequence of the PM-Ca-ATPase. Furthermore, changes in the binding affinities between mutant CaM species in which individual methionines were substituted with glutamine have been previously shown to accurately reflect molecular interactions between CaM and either smooth muscle MLCK or CaM-dependent protein kinase IIα (*12*, *14*), suggesting that the site-directed modification of methionine to glutamine is an effective strategy to identify the role of specific methionines in promoting the productive association between CaM and the CaM-binding sequence within the PM-Ca-ATPase necessary for enzyme activation.

Table 1: Activation of PM Ca-ATPase by CaM Mutants[a]

sample	mutation sites	$[CaM]_{1/2}$ (nM)	k_1 (nM)	k_2 (nM)
CaM	none	1.3 (0.6)	10 (3)	0.2 (0.1)
Q145	M145Q, Y99W	1.4 (0.6)	20 (10)	0.1 (0.1)
Q124	M124Q, Y99W	7.0 (0.6)	40 (10)	0.3 (0.1)
Q144	M144Q, Y99W	13 (2)	70 (30)	0.2 (0.1)
C-2Q	M144Q, M145Q, Y99W	8.4 (0.6)	50 (10)	0.3 (0.1)
C-3Q	M124Q, M144Q, M145Q, Y99W	23 (2)	90 (30)	0.5 (0.1)
N-2Q	M71Q, M72Q, Y99W	5.8 (0.6)	50 (10)	0.1 (0.1)
N-4Q	M36Q, M51Q, M71Q, M72Q, Y99W	28 (3)	130 (30)	0.3 (0.1)

[a] $[CaM]_{1/2}$ is the CaM concentration necessary for half-maximal activation of the PM-Ca-ATPase; k_1 and k_2 correspond to the dissociation constants for the sequential binding of the two globular domains of CaM and were obtained from a least-squares fit to the data in Figure 1, as previously described (*30*).

Construction of Site-Directed Mutants of CaM. Eight mutants of CaM were constructed in which variable numbers of methionines in either the amino- or the carboxyl-terminal domains were substituted with glutamines (Table 1). These mutations involved (i) three mutants in which Gln was separately substituted for three individual methionines in the carboxyl-terminal domain [i.e., Met^{124} (M124Q), Met^{144} (M144Q), Met^{145} (M145Q)]; (ii) three mutants involving the substitution of multiple methionines in the carboxyl-terminal domain with glutamines involving two (i.e., Met^{144} and Met^{145}; C-2Q), three (i.e., Met^{124}, Met^{144}, and Met^{145}; C-3Q), or four (i.e., Met^{109}, Met^{124}, Met^{144}, and Met^{145}; C-4Q) methionines; and (iii) two mutants involving the substitution of multiple methionines in the amino-terminal domain involving two (i.e., Met^{71} and Met^{72}; N-2Q) or four (i.e., Met^{36}, Met^{51}, Met^{71}, and Met^{72}; N-4Q) methionines. In all cases, mutants were also constructed in which Trp was substituted for Tyr^{99} to introduce a fluorescence signal whose emission spectrum is sensitive to possible alterations in the tertiary structures of the carboxyl-terminal domain in these mutants (see below). The introduction of Trp^{99} does not affect the function of CaM (Figure 1), and the large spatial separation between Trp^{99} and all nine methionine side chains in vertebrate CaM avoids ambiguities associated with the interpretation of the intrinsic fluorescence signals in native CaM that are dominated by Tyr^{138} and may therefore result from alterations in short-range quenching interactions that result from the substitution of the proximal carboxyl-terminal methionines (i.e., Met^{144} or Met^{145}) with Gln (*28*). In contrast, Tyr^{99} is greater than 12 Å from any methionine side chain in the crystal structure of CaM (*5*, *29*), suggesting that Trp at this position should accurately reflect global structural

changes that may result from the substitution of individual glutamines with methionine.

Native and mutant CaMs were expressed in *E. coli* strain JM109(DE3), and except for C-4Q all mutants were purified using phenyl-Sepharose hydrophobic chromatography and weak anion exchange HPLC, as previously described (*19*, *20*). The ability of the majority of the CaM mutants to bind to the phenyl-Sepharose resin in a calcium-dependent manner indicates that the structural changes associated with calcium-activation are retained, suggesting that these site-directed mutations do not result in large global alterations in the tertiary structure of CaM. Thus, it is possible to investigate the contribution of individual methionine side chains with respect to the activation of the PM-Ca-ATPase. The inability of C-4Q to bind to the hydrophobic resin suggests that it would have a weak binding affinity for target proteins, and this mutant was not studied in any additional detail. These latter results are consistent with previous suggestions regarding the importance of the carboxyl-terminal domain in CaM in promoting the productive association between CaM and target proteins (*8*).

The possibility that methionines in CaM may be oxidatively modified during the purification of CaM has the potential to compromise the ability to activate the PM-Ca-ATPase (*16*, *17*, *25*). We have therefore used electrospray ionization mass spectrometry (ESI-MS) to measure the average mass of all CaM mutants, confirming the site-directed mutations and ensuring that no posttranslational modifications occur during purification. A single ion current corresponding to the average mass of all CaM mutants within 3 Da of the expected mass, was observed for all mutants, indicating that any observed alterations in function are the result of the introduced site-specific amino acid substitutions rather than possible oxidative modifications that may occur during purification. Thus, these mutant CaMs can be used reliably to investigate the role of individual methionines in promoting the activation of the PM-Ca-ATPase.

Tertiary Structures of CaM Mutants. Prior to a consideration of the effects of the site-directed substitution of any amino acid on protein function, it is important to ensure that the mutation does not have a deleterious effect on protein structure. We have therefore assessed possible global changes in the structure of the carboxyl-terminal domain of CaM mutants whose apparent affinity for the PM-Ca-ATPase is diminished through a consideration of the fluorescence emission spectrum of Trp^{99}, which has previously been shown to be sensitive to the structural uncoupling of the opposing globular domains of CaM (*30*). Changes in the fluorescence intensity of some CaM mutants suggest that the substitution of methionines by glutamines can result in alterations in the local environment around Trp^{99} (Table 2). However, irrespective of the amino acid substitution, there are no significant differences in the fluorescence emission maximum (Table 2), which is sensitive to alterations in the protein fold (*31*). These latter results are consistent with the observation that all CaM mutants are able to fully activate the PM-Ca-ATPase (see below), and indicate that the substitution of Met → Gln does not substantially alter the tertiary structure of the carboxyl-terminal domain of CaM. Furthermore, previous measurements of the magnitude of the changes in the apparent affinity between CaM and target proteins with known crystal structures (i.e., either smooth muscle MLCK or CaM-dependent protein kinase IIα) can be explained in terms of the loss of specific binding interactions in the crystal structure (*12*), indicating that the structural interactions between CaM and these target proteins are similar to those of the native structures. Thus, alterations in the apparent binding affinity between CaM mutants and the PM-Ca-ATPase are expected to provide an accurate measurement of the relative role specific methionines in CaM play in the activation of the PM-Ca-ATPase.

Table 2: Fluorescence Spectral Changes of Trp^{99} in Apo- and Calcium-Activated CaM Mutants[a]

	+EGTA		+calcium	
sample[b]	intensity	λ_{max} (nm)	intensity	λ_{max} (nm)
CaMW	1.00 ± 0.01	355 ± 2	0.95 ± 0.01	351 ± 2
Q144	1.11 ± 0.01	356 ± 2	1.13 ± 0.01	352 ± 2
Q124	0.81 ± 0.01	354 ± 2	1.19 ± 0.02	351 ± 2
C-2Q	0.87 ± 0.01	351 ± 2	1.26 ± 0.02	351 ± 2
C-3Q	0.73 ± 0.01	355 ± 2	1.26 ± 0.02	354 ± 2
N-2Q	1.02 ± 0.03	353 ± 2	0.99 ± 0.01	356 ± 2
N-4Q	1.00 ± 0.01	353 ± 2	0.97 ± 0.01	356 ± 2

[a] Experimental conditions involved 6 μM CaM in 0.1 M HEPES (pH 7.5), 0.1 M KCl, and 0.1 mM EGTA in the absence or presence of 0.2 mM $CaCl_2$. Excitation was at 297 nm using 4 nm bandwidths on both excitation and emission monochromators. Intensity represents the integrated area of each spectrum, and the errors are from two separate measurements. Temperature was 25 °C. [b] Samples are as described in Table 1.

CaM-Dependent Activation of the PM-Ca-ATPase. The ability of CaM mutants to activate the PM-Ca-ATPase was assessed to determine the role of methionines in the amino- and carboxyl-terminal domains in the productive association and activation of the PM-Ca-ATPase. Upon substitution of Met^{145} with Gln, there was no difference in the concentration-dependence of the CaM-dependent activation of the PM-Ca-ATPase relative to that observed for native CaM (Figure 1A; Table 1). In contrast, substitutions of either Met^{124} or Met^{144} with Gln result in a respective 5- and 10-fold increase in the amount of CaM necessary for half-maximal activation of the PM-Ca-ATPase, suggesting that these hydrophobic side chains normally stabilize the association between CaM and the PM-Ca-ATPase. However, the maximal CaM-dependent activation of the PM-Ca-ATPase was unchanged by these site-directed substitutions of Gln for Met, suggesting that decreased hydrophobic interactions between individual methionines and the CaM-binding sequence do not affect the productive association between CaM and the PM-Ca-ATPase necessary for enzyme activation. These latter results are in contrast to previously reported decreases in the ability of CaM to fully activate other target proteins upon substitution of either Met^{124} or Met^{144} with Gln (*12*, *14*), suggesting that in comparison to either smooth muscle MLCK or CaM-dependent protein kinase IIα (whose high-resolution structures between CaM and peptides corresponding to the CaM-binding sequences of these proteins are available) there are substantial differences with respect to the binding mechanisms between CaM and the PM-Ca-ATPase.

To further investigate the role of methionine side chains with respect to the productive interaction between CaM and the CaM-binding sequence in the PM-Ca-ATPase, we have used site-directed mutagenesis to substitute multiple methionines with glutamines in the carboxyl-terminal domain of CaM. Irrespective of the number of methionines in the

carboxyl-terminal domain of CaM, there is no diminution in the maximal CaM-dependent activation of the PM-Ca-ATPase (Figure 1B). The substitution of Met^{124}, Met^{144}, and Met^{145} (i.e., C-3Q) with Gln results in an 18-fold increase in the amount of CaM necessary for half-maximal activation of the PM-Ca-ATPase (i.e., $[CaM]_{1/2}$), indicating that the losses of hydrophobic interactions involving methionine side chains are additive (Table 1). Likewise, the substitution of two (i.e., Met^{71} and Met^{72}; N-2Q) or four methionines (i.e., Met^{36}, Met^{51}, Met^{71}, Met^{72}; N-4Q) in the amino-terminal domain of CaM results in a 4- and 20-fold increase in $[CaM]_{1/2}$, but does not alter the maximal CaM-dependent activation of the PM-Ca-ATPase observed at saturating CaM concentrations (Figure 1C). Thus, while hydrophobic interactions involving methionine side chains within each of the globular domains of CaM are involved in binding to the CaM-binding sequence of the PM-Ca-ATPase, other side-chain interactions between CaM and the CaM-binding sequence are sufficient for enzyme activation at saturating CaM concentrations.

Determination of Binding Affinities between the CaM Domain and the PM-Ca-ATPase. The initial binding interaction between CaM and many target proteins, including the PM-Ca-ATPase, involves the high-affinity association of the carboxyl-terminal domain of CaM with a CaM-binding sequence in the Ca-ATPase that contains a highly conserved aromatic amino acid residue (*8*, *32*). Enzyme activation results from the subsequent association of the amino-terminal domain of CaM with a specific binding site, which is aided by the reduced volume available for diffusion of the amino-terminal domain after the binding of the carboxyl-terminal domain (*30*, *33*, *34*). Therefore, to quantify the influence of glutamine substitutions for individual methionines with respect to the ability of CaM to activate the PM-Ca-ATPase, we have analyzed the CaM-dependent activation of the PM-Ca-ATPase to determine the binding affinities of the opposing globular domains for the binding sites on the PM-Ca-ATPase (Table 1). For CaM binding to the PM-Ca-ATPase, the dissociation constants indicate that following high-affinity association of the carboxyl-terminal domain of CaM with the PM-Ca-ATPase ($k_1 = 10 \pm 3$ nM), the amino-terminal domain of CaM rapidly associates as a result of the increase in effective concentration ($k_2 = 0.2 \pm 0.1$ nM). It should be noted that the actual dissociation constant for the amino-terminal domain upon correction for the reduced volume available following association of the carboxyl-terminal domain to the PM-Ca-ATPase is approximately one million times larger than that determined from this calculation (*33*, *34*). Thus, it is apparent that modest changes in the affinity of the amino-terminal domain of CaM will have little effect on the equilibrium binding constant (i.e., k_2), and that the low affinity of the amino-terminal domain prior to association of the carboxyl-terminal domain ensures the ordered binding between the opposing globular domains of CaM with the PM-Ca-ATPase necessary for enzyme activation. Upon fitting the activation data obtained using the various CaM mutants, we find that the primary effect of the substitution of methionines with glutamines in either domain of CaM is to decrease the initial association (i.e., k_1) between CaM and the PM-Ca-ATPase (Table 1). Thus, methionines located in both amino- and carboxyl-terminal domains of CaM contribute to high-affinity binding. This result is consistent with earlier suggestions that the initial association between CaM and target peptides is guided by nonspecific interactions involving both domain elements in CaM (*35*).

DISCUSSION

Summary. Methionine side chains in CaM stabilize the association between calcium-activated CaM and the PM-Ca-ATPase, as indicated by the increased concentrations of CaM necessary for activation of the PM-Ca-ATPase upon substitution of Gln for the majority of the methionines in either the amino- or the carboxyl-terminal domains of CaM (Figure 1). However, while substitution of Gln for Met at positions 124 and 144, respectively, results in a 5- and 10-fold increase in the amount of CaM necessary for half-maximal activation of the PM-Ca-ATPase, substitution of Met^{145} with Gln (Q145) does not alter the apparent affinity between CaM and the PM-Ca-ATPase (Table 1). Thus, in contrast to other known target proteins (i.e., smooth muscle MLCK, CaMKII, and CaMKIV), Met^{145} does not contribute to the hydrophobic interactions that stabilize the complex between CaM and the PM-Ca-ATPase. CaM can fully activate the PM-Ca-ATPase upon substitution of the multiple methionines with the polar amino acid glutamine (Figure 1). Thus, productive binding of CaM to the PM-Ca-ATPase does not require hydrophobic interactions between methionine side chains in either globular domain of CaM and the CaM-binding sequence of the PM-Ca-ATPase. In contrast, single methionine substitutions with glutamine can result in substantial decreases in the maximal extent of CaM-dependent activation for other target proteins (i.e., smooth muscle MLCK, CaMKI, CaMKII, and CaMKIV) in the presence of saturating CaM concentrations (*12*, *14*). Thus, the PM-Ca-ATPase may be unique, since interactions between methionines in CaM and the binding sites on the Ca-ATPase are not critical for the activation of the PM-Ca-ATPase.

Hydrophobic Interactions and Target Recognition. The indole ring within the CaM-binding sequence of a range of different target proteins, including the PM-Ca-ATPase, has been suggested to represent an important recognition site associated with CaM binding (*2*, *8*, *32*, *39*, *40*). Upon initial binding of the carboxyl-terminal domain of CaM to the indole ring in the CaM-binding sequence of the target protein, the proximity of the amino-terminal domain to the CaM-binding sequence has been suggested to facilitate rapid binding to a range of different target proteins that facilitates enzyme activation (*33*, *34*, *41*). Methionine side chains within each of the hydrophobic binding clefts in CaM have been suggested to be critical to the ability of CaM to bind and activate a range of different target proteins whose CaM-binding sequences possess little sequence homology (*6*, *8*, *12*, *36*). However, while CaM binding is known to stabilize the α-helical conformation of a range of different CaM-binding sequences (*9*−*11*, *42*), it has been unclear whether hydrophobic interactions between methionine side chains and the CaM-binding sequence of the PM-Ca-ATPase are necessary for enzyme activation. Previous results indicate that the substitution of individual methionines with glutamines results in a decreased apparent affinity between CaM and smooth muscle MLCK, CaMKI, or CaMKII that correlates with the number of hydrophobic contact interactions between methionine side chains in CaM and side chains within these target sequences (*12*, *14*). In some cases (e.g., Q124), there

are corresponding decreases in the CaM-dependent activation of smooth muscle MLCK, CaMKI, CaMKII, and CaMKIV (*12*, *14*), suggesting that hydrophobic contact interactions between methionine side chains and CaM-binding sequences in target proteins can be important to the mechanism of enzyme activation. In the case of the PM-Ca-ATPase, the substitution of the majority of the methionine side chains in the amino- or carboxyl-terminal domains of CaM results only in changes in the apparent affinity between CaM and the PM-Ca-ATPase, with no change in the maximal extent of activation. Thus, interactions between methionine side chains in CaM and the CaM-binding sequence of the PM-Ca-ATPase are not critical to inducing structural changes within the CaM-binding sequence involved in the activation of the PM-Ca-ATPase, and appear to be primarily involved in modulating the affinity of CaM binding. Thus, the suggestion that methionine side chains in the carboxyl-terminal domain of CaM form a hydrophobic anchor critical to protein activation is not true for the PM-Ca-ATPase. These results are consistent with earlier suggestions that the majority of the interactions that define the binding specificity between CaM and the CaM-binding sequences in target proteins associated with enzyme activation do not necessarily involve hydrophobic interactions (*37*, *38*).

The major effect of substituting glutamines for the methionines in either the amino- or the carboxyl-terminal domain of CaM is to reduce the initial binding interaction (i.e., k_1) between CaM and the PM-Ca-ATPase, which has previously been suggested to primarily involve elements within the carboxyl-terminal domain (*8*, *33*, *34*). The secondary binding of the opposing globular domain (i.e., k_2), which results in enzyme activation, is relatively insensitive to the substitution of glutamines for methionines in any of the CaM mutants (Table 1). The large decreases in the affinities between the C-3Q and N-4Q mutants of CaM relative to native CaM for the CaM-binding sequence of the PM-Ca-ATPase suggest a role for methionine side chains in both globular domains in mediating the initial binding of CaM to the PM-Ca-ATPase. The ability of these mutants to fully activate the PM-Ca-ATPase at saturating CaM concentrations is therefore consistent with the suggestion that distinct molecular interactions may be involved in the binding and activation of different target proteins by CaM (*8*, *13*, *36*, *39*, *43*). However, since the binding of CaM to target peptides involves a fast association step and a slower rearrangement step (*39*), it is also possible that methionine side chains are critical to the stabilization of intermediate structures associated with enzymatic activation of the PM-Ca-ATPase. Furthermore, methionines have been suggested to play an important role in the stabilization of the open conformation of the binding clefts in CaM (*2*), suggesting that it is also possible that the substitution of multiple methionines in either domain with glutamines results in a reduction in the fractional time each domain spends in the open conformation necessary for target association, resulting in a reduction in the binding affinity.

Relationship to Other Studies. While many CaM mutations have been reported that result in an inability to activate target enzymes fully in the presence of saturating CaM concentrations (*12*−*14*, *36*, *44*−*48*), it has been unclear whether these functional effects are due to the selective loss of specific binding interactions involved in enzyme activation or the result of global structural changes that alter the binding mechanisms between CaM and target proteins. Similar decreases in the extent of enzymatic activation of some target enzymes are observed upon chemical cross-linking of the isolated amino- and carboxyl-terminal CaM domains with a flexible linker (*49*), suggesting that global structural changes that alter the spatial arrangement of the opposing globular domains of CaM may result in an altered binding interaction that fails to induce enzyme activation. The current results suggest that the substitution of polar glutamine for methionine has no effect on the ability of CaM to fully activate the PM-Ca-ATPase, indicating that hydrophobic binding interactions between CaM and the CaM-binding sequence of the PM-Ca-ATPase are not necessary for enzyme activation. Thus, a decreased ability to activate the PM-Ca-ATPase would necessarily involve the loss of other binding interactions between CaM and the CaM-binding sequence that may be the result of global conformational changes.

Conclusions and Future Directions. The hydrophobic interactions between CaM and the PM-Ca-ATPase involved in binding and enzymatic activation are distinct from those described for other CaM-dependent enzymes. Thus, Met^{145} plays no role in binding, and methionine side chains are not critical to the maximal CaM-dependent enzymatic activation of the PM-Ca-ATPase. However, methionine side chains are critical in defining the affinity of the interaction between CaM and the PM-Ca-ATPase, and upon substitution of multiple methionines with glutamine in either the amino- or the carboxyl-terminal domains of CaM, there is a similar reduction in the binding affinity between CaM and the PM-Ca-ATPase necessary for enzyme activation. Future studies aimed at understanding the mechanism of activation of the PM-Ca-ATPase by CaM will require direct measurements of the structural transitions in CaM and the CaM-binding sequence of the PM-Ca-ATPase that result in enzymatic activation.

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