Characterization of Substrate Phosphorylation and Use of Calmodulin Mutants to Address Implications from the Enzyme Crystal Structure of Calmodulin-dependent Protein Kinase I*

(Received for publication, June 11, 1997, and in revised form, September 19, 1997)

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Calcium/calmodulin (CaM) directly activates CaM-dependent protein kinase I (CaMKI) by binding to the enzyme and indirectly promotes the phosphorylation and synergistic activation of CaMKI by an exogenous kinase. We have evaluated the initial CaMKI-dependent activation of the unphosphorylated form of CaMKI. The kinetics of bacterially expressed human CaMKI show that the peptide syntide-2 is a relatively poor substrate, whereas the synapsin site-1 peptide is 17-fold more specific. The peptide ADR1G is 400-fold more specific than syntide-2, and its catalytic rate is among the highest reported for a kinase peptide substrate. To understand how CaM activates CaMKI, we have characterized the activation of the enzyme by CaM mutants with substitutions at hydrophobic residues. The point mutant M124Q located in the C-terminal domain of CaM produced a 57-fold increase in the CaM activation constant for CaMKI and suggests the involvement of methionine 124 in an important hydrophobic interaction with tryptophan 303 of CaMKI. Substituting two, three, and five hydrophobic residues in the N-terminal domain of CaM increased the CaM activation constant for CaMKI by 10–190-fold and lowered the maximal enzyme activity by more than 80%. Two of these N-terminal mutants of CaM do not affect the $K_m$ for peptide substrate but instead produce a 5–10-fold higher $K_m$ for ATP. This result demonstrates the critical role of the N-terminal domain of CaM in regulating the access of ATP to CaMKI.

The serine/threonine class of protein kinases are regulated by a variety of second messengers such as cyclic nucleotides, diacylglycerol, and calcium ions (Ca$^{2+}$). These substances activate kinases by a similar mechanism that involves releasing the autoinhibited state of the enzyme to allow access of substrate to the active site (1). The group of protein kinases regulated by Ca$^{2+}$ and the ubiquitous, eukaryotic protein calmodulin (CaM)$^1$ conform to this mode of regulation. The binding of Ca$^{2+}$ by CaM enables the Ca$^{2+}$/CaM complex to recognize, bind to, and activate different classes of intracellular target enzymes including the Ca$^{2+}$/CaM-regulated protein kinases. The x-ray crystal structure of Ca$^{2+}$/CaM shows a 148-residue dumbbell shaped protein consisting of two homologous domains separated by a short linker peptide (2). Other structural studies demonstrate that Ca$^{2+}$/CaM forms 1:1 complexes with peptides whose sequences correspond to the CaM binding domains of the smooth muscle myosin light chain kinase (smMLCK) (3), skeletal muscle myosin light chain kinase (skMLCK) (4), and CaM kinase IIα (CaMKII) (5). The peptides fold into a helix and directly interact in an antiparallel orientation (N to C) with two hydrophobic pockets in each domain of CaM. The relevance of these peptide models to the interaction of CaM with the intact protein kinases has been validated by mutagenesis studies on CaM and the protein kinases (6–9).

Ca$^{2+}$/CaM-dependent protein kinase I (CaMKI) is another member of the family of CaM-regulated kinases. Human CaMKI is a 370-residue, ~41-kDa protein that is expressed in numerous tissues (10). CaM regulates the activity of CaMKI in two ways. First CaM can activate CaMKI by the conventional method of directly binding to the enzyme and relieving the autoinhibited state. Secondly the binding of CaM enables CaMKI to be phosphorylated at Thr177 by an exogenous kinase that further enhances the CaM-dependent activity of the enzyme (10–15). Previous characterizations of CaMKI used enzyme purified from mammalian tissues that contain CaM and CaM kinase kinases (16–19) or were activated by the addition of an exogenous CaM kinase kinase preparation (10–14). Recently the cloning and bacterial expression of rat and human CaMKI as glutathione S-transferase (GST) fusion proteins (GST-CaMKI) have allowed the isolation of the unactivated form of the enzyme (10, 20, 21). This has led to a characterization of the regions of CaMKI involved in autoinhibition, CaM binding, and phosphorylation by CaM kinase kinase. However, a detailed analysis of the enzyme kinetics or mechanism of direct activation by CaM of the unphosphorylated form of CaMKI has yet to be undertaken.

In this study we characterize the substrate phosphorylation and CaM-dependent activation of unphosphorylated human GST-CaMKI. Experiments on tissue-purified CaMKI demonstrated that the enzyme poorly phosphorylated most proteins tested but preferred substrates also phosphorylated by the cAMP-dependent protein kinase such as synapsin I (12), the cAMP response element binding protein (22), and the cystic fibrosis conductance regulator (23). To characterize the enzyme kinetics of the unphosphorylated form of CaMKI we have compared the phosphorylation of two known peptide substrates of CaMKI, synapsin site-1 (12) and syntide-2 (21), with that of a peptide derived from the yeast transcriptional activator of the alcohol dehydrogenase gene (ADR1) (24). The ADR1 peptide was chosen because it was a superb substrate for cAMP-de-
ependent protein kinase (25) and also because it had been proven to be a very good substrate for two other members of the CaM kinase family, CaM kinase II and CaMKIV (26).

The solution of the x-ray crystal structure of bacterially expressed rat CaMKI (residues 1–320) revealed how the CaM binding sequence of CaMKI (residues 300–316) autoinhibits the enzyme by blocking the access of substrates (27). We felt that two unusual aspects of the CaM binding domain of CaMKI merited special consideration. The first is the exposure to solvent of a large hydrophobic residue, Trp303, which was proposed by Goldberg et al. (27) to play an important role in CaM recognition. The second is the interaction of CaMKI residues 307–316 with part of the ATP binding loop of the enzyme, thus implicating CaM in regulating the access of ATP. In the three-dimensional structures of CaM-peptide complexes, the antiparallel orientation of CaM with the CaM binding peptides indicates that CaM might interact with the CaM binding domain of CaMKI in a similar manner (Fig. 1). These structures predict that Trp303 of CaMKI would interact with the C-terminal hydrophobic domain of CaM, whereas residues 307–316 of CaMKI would primarily interact with the N-terminal hydrophobic domain of CaM. To test these predicted interactions we have investigated the activation of CaMKI by CaM with various substitutions at hydrophobic residues in its two domains.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, dithiothreitol, glutathione, Heps, isopropyl-β-D-thiogalactopyranoside, and Tween 80 (polyoxyethylene sorbitan monooleate) were purchased from Sigma. Protease inhibitors and ATP were from Boehringer Mannheim. (g-32P)ATP was obtained from Amersham. Calcium chloride and magnesium chloride were from Fisher. Glutathione-Sepharose-4B was purchased from Pharmacia Biotech Inc. Syntide-2 (PLARTLSVAGLPGKK) was purchased from Sigma. A variant of the original synapsin site-1 peptide in which Ala was substituted at the P1 position for the original Ser residue (LRRRLSDDANG) (12), and ADR1G (LKKLTRASFGQQ) were prepared as C-terminal amidase by automated solid phase peptide synthesis. Peptide analogs of ADR1 used in preliminary studies were the kind gift of Dr. Bruce E. Kemp from the St. Vincent’s Institute (Holt Laboratory, Melbourne, Australia).

Expression and Purification of Protein—Full-length GST-CaMKI was expressed in BL-21 bacteria by isopropyl-β-D-thiogalactopyranoside induction and purified at 4 °C by single step glutathione affinity chromatography as described previously (10). The purified fusion protein was stored in 40% glycerol at −80 °C. CaMKIV was expressed in s9 cells and purified as described (26). Wild type and mutant CaM proteins were individually expressed by heat induction in N5151 bacteria and purified to homogeneity by a two-step hydrophobic interaction and gel filtration chromatography procedure as described previously (6, 7). Concentrations of protein stock solutions were determined by the Bradford assay (28) using bovine serum albumin protein standards for the kinase assays and CaM protein standards for CaM assays.

Assays of CaM-dependent Activation of CaM Kinases—Peptide phosphorylation assays were performed at 30 °C in a standard solution consisting of 50 mM Heps (pH 7.5), 10 mM MgCl2, 1 mM CaCl2, 1 mM dithiothreitol, 0.1% Tween 80, 0.5 mg/ml bovine serum albumin, and various concentrations of CaM, ATP (~50cpm/mmol), and peptides in a final volume of 50 μl. The reactions were initiated by the addition of enzyme to a final concentration of 1 nm and terminated after 5 or 10 min. Autophosphorylation and autoactivation of GST-CaMKIV was negligible under these conditions (10), and the rate of phosphorylation of peptides was linear up to 15 min, using in most cases <5% of substrate. Reactions were terminated by spotting 40 μl on Whatman P-81 phosphocellulose filters followed by extensive washing in 75 mM phosphoric acid. The dried filters were counted on a Beckman LS 6000 scintillation counter. Kinetic data from substrates of CaMKI were determined using a single plotting arrangement ranging from 0.2 to 5 mM ATP and peptide concentrations ranging from 0.05 to 4 μM in some cases. The concentrations of wild type CaM in these assays was saturating at 5 μM, whereas kinetics for the N-terminal domain CaM mutant proteins were performed at 20 μM CaM. Kinase assays at increasing concentrations of wild type or mutant CaM protein were performed at fixed 0.4 mM concentrations of ATP and 0.2 mM ADR1G. The concentration of ATP stock solution was determined spectrophotometrically (ε206 = 14,900 M−1 cm−1). The concentrations of substrate stock solutions were determined from amino acid analysis and in applicable cases spectrophotometrically based on the extinction coefficient for phenylalanine (ε280 = 200 M−1 cm−1).

Analysis of Kinetic Constants—Kinetic constants of substrates were estimated graphically from linear regression analysis of double reciprocal primary and secondary rate plots as described for a general two substrate reaction (29). Activation constants and the percentage of maximal activity for wild type CaM and CaM mutant protein were derived as described previously (6).

RESULTS

The aim of this work was to characterize the basal CaM-dependent activation of unphosphorylated CaMKI. Previous studies had demonstrated that the bacterially expressed human enzyme was in the basal, unphosphorylated state because it was capable of additional activation due the phosphorylation of Thr177 by an exogenous CaM kinase kinase (10). Because human CaMKI is 97% identical to the rat enzyme, it was used as a model to study implications of the three-dimensional crystal structure of rat CaMKI residues 1–320 (27). CaMKI was expressed as an N-terminal GST fusion protein in bacteria and purified to >95% homogeneity by single step affinity chromatography. SDS-polyacrylamide gel electrophoresis analysis of the 67-kDa GST-CaMKI revealed a minor contaminating protein fragment (~5%) that was the same size as GST (~26 kDa).

We initially conducted a limited survey of peptides to identify the most specific substrate available, i.e. the most efficiently phosphorylated with the highest kcat/Km ratio. The three peptides tested were syntide-2, synapsin site-1, and a peptide derived from ADR1. Gly was substituted for Ala at the P1 position of the original ADR1 sequence, referred to as ADR1G, based on the higher rate of phosphorylation of ADR1G by CaMKIV in comparison with the original ADR1 peptide (26).

The kinetic constants for peptide phosphorylation by CaMKI are presented in Table I. These results demonstrate that the Km of CaMKI for ATP in the presence of either synapsin site-1 or ADR1G was 8-fold lower than with syntide-2. The Km for the peptides themselves exhibited even larger differences, with syntide-2 exhibiting the highest Km, synapsin site-1 exhibiting an intermediate Kms, and ADR1G having the lowest Kms for the enzyme. The kcat values, derived at increasing concentrations of peptide substrate, revealed that ADR1G had the highest turnover, syntide-2 was lower by about 8-fold, and synapsin site-1 had an intermediate rate. The efficiency of catalysis (kcat/Km) ranged 3 orders of magnitude as synapsin site-1 and ADR1G were 17- 400-fold, respectively, more specific than syntide-2. Because ADR1G was by far the most preferred of the three peptides tested, it was used in subsequent experiments with CaM mutants.

A comparison of the CaM binding domain of CaMKI with those from other CaM-regulated kinases indicated that the N-terminal domain of CaM interacts with residues 309–319 of CaMKI (Fig. 1). In the crystal structure of CaMKI these
residues also interact with the ATP binding loop. This observation indicated that CaM might facilitate ATP binding to the enzyme. To investigate the role of the CaM N-terminal domain in the activation of CaMKI, substitutions were introduced in the N-terminal hydrophobic pocket of CaM. Five nonpolar residues on CaM that interact with the smMLCK CaM binding peptide (4) were chosen for study because the shortening of these side chains had affected the activation of two other CaM-regulated enzymes, smMLCK and CaMKIIα (7). Three mutant CaM proteins were created by decreasing the size of either two side chains (M51A,V55A), three side chains (L32A,M51A,V55A) or five side chains (L32A,M51A,V55A,F68L,M71A) in the N-terminal domain of CaM. We then compared the ability of these mutant CaM to activate CaMKI and the closest related kinase, CaMKIV. The N-terminal domain mutants of CaM produced dramatic differences in the activation profiles for CaMKI (Fig. 2A). The double mutant of CaM (M51A,V55A) led to a 10-fold increased activation constant ($K_{CaM}^*$) over the wild type CaM (10 nM), whereas the triple and quintuple mutants had considerably higher increases at 74- and 190-fold, respectively. The double CaM mutant also activated CaMKI to 90% of maximal activity, whereas the triple mutant resulted in approximately 52% and the quintuple mutant achieved only 17% of maximal activity. In contrast with CaMKI, however, all three CaM mutant proteins were capable of maximal (100%) activation of CaMKIV (Fig. 2B). The double and triple substitution mutants had a similar $K_{CaM}^*$ values as wild type CaM, whereas the $K_{CaM}^*$ of the quintuple mutant was almost 10-fold higher. Table II summarizes the activation parameters for these three CaM mutants.

To understand the mechanism underlying the decreased activation of CaMKI by the CaM N-terminal domain mutants, we tested whether they affected the kinetics of either ATP or ADR1G binding to CaMKI. The results from an analysis of CaMKI activity in the presence of saturating concentrations of wild type CaM or the triple and quintuple CaM mutant proteins (Table III) demonstrate that the triple mutant resulted in a 5-fold increase in the $K_m$ for ATP, whereas the quintuple mutant had a 10-fold increase. In contrast, neither CaM mutant had an appreciable effect on the $K_m$ for ADR1G. These results provide compelling evidence for an important role of the N-terminal domain of CaM in regulating the accessibility of the enzyme for ATP.

The unusual solvent exposure of Trp303 in the three-dimensional structure of CaMKI implied the presence of a complementary hydrophobic binding surface on Ca$^{2+}$/CaM. To investigate the roles of different hydrophobic microenvironments of CaM on the binding of CaMKI, we measured the activation of GST-CaMKI by CaM point mutants where a polar Gln was substituted for each of nine hydrophobic Mets at the wide-spread positions 36, 51, 71, 72, 76, 109, 124, 144, and 145 of CaM. The Mets were chosen for study because they directly interact with CaM binding peptides (3–5) and are also functionally important for the binding and activation of three related CaM-dependent protein kinases (6). The activation constants and the percentage of maximal activity for each of the nine Met point mutants of CaM are presented in Table IV. An evaluation of the percentage of maximal activation of CaMKI by the nine individual mutants of CaM allows them to be separated into two major groups. The first group consisting of M36Q, M51Q, M72Q, M76Q, M109Q, M144Q, and M145Q achieved 90–100% of maximal activity. The second group consisting of M71Q and M124Q had lower maximal activities at 75 and 70%, respectively.

The activation constants ($K_{CaM}$) for each of the nine CaM Met mutants are presented as a function of the CaM secondary structure in Fig. 3 with the sequence positions listed above each residue. Starting with the N-terminal domain of CaM (residues 1–75), the $K_{CaM}$ of Met mutants M36Q, M71Q, and M72Q have similar activation constants, which are within 3–5-fold that of the wild type protein ($K_{CaM} = 10$ nM). The M51Q
substitution resulted in the largest $K_{CaM}$ in the N-terminal domain, which was more than 10-fold greater than the wild type. The M76Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M76Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type. The M124Q mutant located between the two CaM domains (residues 76–83) was identical in behavior to the wild type.

DISCUSSION

One of the goals of this study was to characterize the kinetics of the unphosphorylated form of CaMKI. The high turnover rate ($k_{cat}$) for all three peptide substrates by the Ca$^{2+}$/CaM-activated form of bacterially expressed CaMKI was an unexpected result because previous reports suggested that the phosphorylation of CaMKI by an exogenous kinase was a necessary step in the activation of the enzyme (12–14, 30). A published review of kinase peptide substrates shows that the highest reported $V_{max}$ (31 μmol/min/mg) was for a peptide substrate of the CaM-activated skMLCK (31) and the phosphorylation of the original ADR1 peptide by cAMP-dependent protein kinase was next highest at 28 μmol/min/mg. In comparison the $V_{max}$ for CaMKI ($V_{max} = -0.9 k_{cat}$) obtained in the present study was 12 μmol/min/mg for syntide-2, 25 μmol/min/mg for synapsin site-1, and 101 μmol/min/mg for ADR1G. By these criteria, in the absence of phosphorylation by an exogenous kinase, the CaM-activated form of CaMKI qualifies as a highly active enzyme.

The relatively efficient phosphorylation of ADR1G was especially propitious for our subsequent experiments on CaM mutants and may be due to unique structural determinants in this particular peptide. The P+1 to P+4 region of ADR1G may play a role in this effect because the substitution of Gly for Ala at P+3 of the original ADR1 sequence resulted in a higher rate of catalysis for CaMKIV (26). The importance of the P+1 to P+4 region on the phosphorylation of peptides has been noted in the case of Ca$^{2+}$/CaM-dependent skMLCK (32). The higher $K_m$ of synapsin site-1 in comparison with ADR1G may be due to a previously noted negative determinant Asp at P+1 of synapsin site-1 (12). In addition, when C-terminal and N-terminal CaMs were tested and exhibited a 57-fold increase over wild type. M124Q in the C-terminal domain had the largest effect of any of the nine mutants tested and exhibited a 57-fold increase in $K_{CaM}$. The M124Q mutant exhibited a 1.5-fold increase over wild type CaM for the $K_m$ of both ATP and ADR1G, respectively (data not shown).

![FIG. 3. $K_{CaM}$ values for methionine to glutamine mutants of calmodulin.](image)

Activation constants ($K_{CaM}$) are presented as a function of the secondary structure of the 148-residue CaM. The CaM structure is depicted as a series of helices (open bars) separated either by connecting peptides or four Ca$^{2+}$ binding loops. The N-terminal domain (residues 1–75) is separated from the C-terminal domain (residues 84–148) by a connecting peptide (residues 76–83). The wild type protein has a $K_{CaM}$ of 10 nM.

detector for peptide binding by CaMKI. However, the exact determinants on ADR1G responsible for its exceptional rate of phosphorylation by CaMKI remain to be elucidated.

Another unexpected finding was the observation that the efficiency of ATP binding by CaMKI depended on the peptide substrate present. This effect contributed to the relatively low levels of phosphorylation of syntide-2 due to the higher $K_m$ for ATP in the presence of this particular peptide. Consequently at low concentrations of reactants (<0.1 mM syntide-2 and < 0.2 mM ATP), the specific activities of the enzyme approached 0.1–0.2 μmol/min/mg enzyme, a result similar to those previously reported for rat GST-CaMKI (21). The reason for this effect of syntide-2 is unclear but may be due to the presence of negative structural determinants on the peptide that interfere with the binding of ATP to the enzyme.

2 D. Chin, K. E. Winkler, and A. R. Means, unpublished observation.
To identify the regions on CaM responsible for binding and activating CaMKI we characterized the activation of GST-CaM by CaM carrying mutations at hydrophobic residues located throughout the molecule. The results from the single substitution of the nine Met to Gln mutations in CaM define regions on CaM involved in binding to CaMKI. Two internally homologous, at position 51 in the N-terminal domain and at position 124 in the C-terminal domain, appear to play a greater role than others. The smaller $K_{CaM}$ of M51Q compared with that of M124Q suggests that the individual N-terminal domain residues of CaM contribute relatively less to the binding of CaMKI than their homologous C-terminal domain counterparts. The solution and crystal structures of Ca$^{2+}$-CaM bound to the CaM binding peptides of skMLCK, smMLCK, and CaMKII demonstrate in each case that Met$^{124}$ directly interacts with a nonpolar residue homologous to CaMKI Trp$^{303}$ (Fig. 1). Therefore, the 57-fold increase in $K_{CaM}$ of M124Q may be due to the interference of this mutation with a hydrophobic interaction between Met$^{124}$ of CaM and Trp$^{303}$ of CaMKI. Such an interaction would support the proposed role of the solvent accessible Trp$^{303}$ as an initial binding site for CaM (27). Indeed a similar role has been proposed for the homologous hydrophobic side chains of other CaM-regulated kinases and enzymes, based on the effects of CaM mutants on smMLCK, CaMKII, and CaMKIV (6).

The results from our study of the CaM mutants also indicated that the N-terminal domain of CaM contributes to a high affinity interaction with CaMKI. The single substitution of Met$^{51}$ and multiple substitutions of 2, 3, or 5 residues in the N-terminal domain of CaM cumulatively increase the $K_{CaM}$ from 10 nM for wild type CaM to 1900 nM for the quintuple mutant. Deletion mutagenesis studies demonstrated that residues 310–321 of the GST-CaM protein are important for binding CaM either in a gel overlay assay or to a CaM-Sepharose column (21). These results from CaMKII complement those from the N-terminal domain mutants of CaM because the truncated CaMKI residues between positions 310 and 321 are predicted to interact with the N-terminal domain of CaM (see Fig. 1).

In contrast to CaMKI, the same N-terminal mutants of CaM had lesser effects on the $K_{CaM}$ Values for CaMKIV. These results suggest that hydrophobic residues in the N-terminal domain of CaM perform different roles in activating different CaM-dependent protein kinases. In the case of CaMKI, the residues of CaM chosen for mutation stabilize a high affinity complex as well as activate the enzyme. CaMKII (7) and CaMKIV (6) show a different form of regulation by CaM because they are not affected by the double and triple mutants of CaM but are both impaired by the quintuple CaM mutant (<10-fold increase in $K_{CaM}$). In contrast to these three enzymes, however, smMLCK binds to all three N-terminal domain mutants of CaM with equally high affinity but exhibits progressively lower enzymatic activity (7).

The lower maximal activity of CaMKI on activation by CaM carrying mutations in its N-terminal domain might be due to a lowered enzyme $k_{cat}$ a high $K_{m}$ for either substrates, or a higher $K_{cat}$ for both substrates. However, the N-terminal domain mutants of CaM interfered with the binding of only one substrate by raising the $K_{m}$ for ATP by more than 10-fold. The finding that the triple and quintuple CaM mutants did not unduly affect the $K_{cat}$ of CaMKI for the ADR1G peptide suggests that these CaMs were still capable of derepressing that portion of the CaMKI autoinhibitor (residues 294–303) proposed to block peptide substrate binding (27). The contrasting effect of the same mutant CaMs on the $K_{m}$ for ATP is consistent with an interaction between the N-terminal domain of CaM and residues 309–319 of CaMKI (Fig. 1). Because in the absence of CaM, residues 307–316 of CaMKI block the ATP binding loop of the enzyme (27), mutations in the N-terminal domain would prevent the proper binding of CaM to residues 309–316 and thus interfere with the access of ATP to CaMKI. This would account for the higher $K_{cat}$ for ATP exhibited by CaMKI in the presence of CaM with multiple mutations in the N-terminal domain. In concert with the demonstrated pseudosubstrate-like autoinhibition of the peptide substrate binding site by CaMKI residues 294–321 (21), this unusual and unprecedented mechanism of restricting the cosubstrate ATP provides an additional method of regulating the activity of the enzyme.

In conclusion the effects of CaM mutants on the activation of full-length human GST-CaMKI are in general agreement with implications from the three-dimensional structure of the truncated rat CaMKI. The results suggest a model in which an initial low affinity interaction occurs between a hydrophobic microenvironment located in the C-terminal domain of CaM that includes Met$^{124}$ and the exposed Trp$^{303}$ of CaMKI. On the basis of the solvent accessibility of Met$^{124}$ in the structures of both Ca$^{2+}$-free CaM and that of Ca$^{2+}$-bound CaM (34), we have proposed that this interaction between the C-terminal domain of CaM and target enzymes may exist at low or resting concentrations of intracellular free Ca$^{2+}$ (6). Recent evidence from the Ca$^{2+}$-dependent interaction of CaM binding peptides with the two isolated domain fragments of CaM is consistent with this hypothesis (35). At elevated levels of Ca$^{2+}$, the next step in the activation process involves the high affinity binding of both domains of CaM to CaMKI, which sequesters the enzyme autoinhibitor. The effects of the CaM mutant M71Q, which lowers CaMKI maximal activity without destabilizing the Ca$^{2+}$/CaM-enzyme complex, suggest that this event may involve short range attractive forces such as van der Waals’ contacts, as was the case for smMLCK (7). The subsequent CaM-induced conformational change liberates the binding sites on CaMKI for both substrates, with the N-terminal domain of CaM promoting the access of ATP to the enzyme. These events allow the unphosphorylated CaMKI to phosphorylate proteins, with preference for substrates that are most efficiently catalyzed such as, for example, ADR1G. Ultimately, the binding of CaM by CaMKI also promotes the phosphorylation and further activation of CaMKI by an exogenous CaM kinase kinase. The implications of this model are being tested with the unphosphorylated and CaM kinase-activated forms of CaMKI.

Acknowledgment—We thank Christine M. Padgett for reviewing the manuscript.

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