A Peptide Model for Calmodulin Trapping by Calcium/Calmodulin-dependent Protein Kinase II*

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Autophosphorylation of Ca\(^{2+}/\)calmodulin-dependent protein kinase II (CaM-kinase) induces a more than 1000-fold increase in calmodulin (CaM)-binding affinity by dramatically decreasing the off-rate for CaM. In this report, we investigate the molecular mechanism for this phenomenon by comparing the rate of dissociation of a novel fluorescently labeled CaM from two synthetic peptides and from the phosphorylated and nonphosphorylated forms of a recombinant preparation of CaM-kinase. Dissociation of a complex of CaM and CKII(296–312), a peptide representing close to the minimum CaM-binding domain of the α subunit of CaM-kinase, exhibited a fast off-rate of 5.0 s\(^{-1}\). This was similar to the off-rate of 1.1 s\(^{-1}\) for the dissociation of CaM from the nonphosphorylated form of CaM-kinase. In contrast, dissociation of CaM from either autophosphorylated CaM-kinase or peptide CKII(290–314) was extremely slow with apparent off-rates of about 3–9 \(\times 10^{-5}\) s\(^{-1}\). Along with information from the crystal structure of CaM(20)/CaM bound to CKII(290–314) (Meador, W. E., Means, A. R., and Quiocio, F. A. (1993) Science 262, 1718–1721), our results suggest a model in which CaM-dependent autophosphorylation of CaM-kinase induces a conformational change in the region of the CaM-binding domain which allows the formation of additional stabilizing interactions with CaM. We predict that this involves amino acids 295–298 in CaM-kinase. The possible consequences of these observations on the reversibility of CaM trapping in native CaM-kinase are discussed.

Ca\(^{2+}/\)calmodulin-dependent protein kinase II (CaM-kinase)\(^3\) is a ubiquitous multifunctional Ser/Thr directed protein kinase. It has numerous roles in mediating cellular responses to increased intracellular Ca\(^{2+}\) such as alterations in neurotransmitter synthesis, ion channel regulation, regulation of cell division, modulation of muscle contractility, and modulation of gene transcription (for review, see Ref. 1). CaM-kinase is activated by binding to the Ca\(^{2+}\) bound form of calmodulin, which dramatically increases the affinity of the enzyme for Mg\(^{2+}/\)ATP, thus leading to substrate phosphorylation and phosphorylation of the enzyme itself (autophosphorylation).

CaM-kinase purified from rat forebrain is a holoenzyme composed of 8–12 α and β subunits found in an approximate ratio of 3:1, respectively. The formation of holoenzymes is essential for rapid subunit autophosphorylation (2, 3). Both inter- and intrasubunit phosphorylation occurs within a holoenzyme, both leading to distinct functional changes in enzyme behavior. Intrasubunit autophosphorylation includes a site identified as Thr\(^{296}\) in the α subunit (2, 3), while intrasubunit autophosphorylation occurs at Thr\(^{308}\) and/or Thr\(^{306}\) in the center of the CaM-binding domain (2, 3). The functional consequences of CaM-kinase autophosphorylation are well studied and include generation of Ca\(^{2+}/\)calmodulin-independent activity (4–8) and inactivation and/or CaM insensitivity (9–13). Recently, Meyer et al. (14) discovered that autophosphorylation of CaM-kinase at Thr\(^{296}\) dramatically increases the affinity of the enzyme by decreasing the rate of dissociation of CaM by more than 3 orders of magnitude. This phenomenon is referred to as CaM trapping.

Two possible biological roles have been proposed for CaM trapping by CaM-kinase (14). The first involves an intrinsic effect on CaM-kinase activity as formalized by Hanson et al. (2). This model predicts that at a certain threshold, increased frequencies of Ca\(^{2+}\) oscillations will produce the autophosphorylated form of CaM-kinase and thus transform the enzyme into the CaM-trapped state. Since CaM then remains bound to the enzyme for longer times, the probability of more subunits entering the trapped state is increased, sustaining a greater proportion of CaM-kinase subunits in the fully active state. A second possible biological role for CaM trapping would be to modulate the availability of free CaM in discrete subcellular locations. CaM-kinase is very abundant in brain, representing as much as 2% of total hippocampal protein (1), and it is likely that its subcellular distribution may be in excess of CaM in certain regions of the neuron where CaM-kinase is postulated to be particularly concentrated, such as postsynaptic densities (15, 16). Conversion of CaM-kinase from a low to a high affinity CaM-binding protein has the potential to redistribute CaM in favor of binding to CaM-kinase during a period of high frequency Ca\(^{2+}\) oscillations and thus decrease the activity of a variety of other CaM-dependent enzymes.

Studies using synthetic peptides (17–21) as well as the crystal structure of CaM bound to a synthetic CaM-kinase peptide (22) provide information concerning the structural basis for enzyme regulation and CaM binding. Amino acids 281–301 in CaM-kinase encompass two inhibitory domains, one (surrounding Thr\(^{286}\)) that binds to the ATP binding site and another (surrounding Lys-291) that binds to the substrate binding site.

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thus inhibiting the enzyme in the absence of Ca\(^{2+}\)/CaM. The minimum CaM-binding domain was defined as amino acids 296–309 of the \(\alpha\) subunit (19). However, the crystal structure of Ca\(^{2+}\)/CaM bound to a synthetic peptide spanning amino acids 290–314 of CaM-kinase (22) shows that amino acid residues 293–310 adopt an \(\alpha\)-helical structure and form stabilizing contacts with Ca\(^{2+}\)/CaM. This suggests that the minimum CaM-binding domain (residues 296–309) does not encompass all amino acids with the potential to interact with CaM.

Proteolysis studies showed that autophosphorylation of Thr\(^{296}\) induces a conformational change in CaM-kinase that leads to exposure of previously cryptic proteolytic sites (23, 24). This observation, coupled with the peptide studies described above, lead us to hypothesize that autophosphorylation of Thr\(^{296}\) exposes residues on the enzyme that make stabilizing contacts with CaM leading to the CaM-trapped state. If this is true, then phosphorylation may only be required for high affinity binding of CaM within the context of native CaM-kinase, and synthetic peptides delineating different regions of the CaM-binding domain could potentially repel the untrapped and trapped forms of the enzyme. In this report, we identify two synthetic peptides that appear to kinetically mimic the untrapped and trapped forms of CaM binding to CaM-kinase, neither of which include the autophosphorylation sequence surrounding Thr\(^{296}\).

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Mutagenesis of Calmodulin**—A bacterial expression plasmid for CaM which uses the \(\lambda\) phage P\(_2\) promoter was generated by subcloning a \(BglII/\lambda\)coI fragment from pT754NCoI (25) into the plasmid pCM23N (26) which had been digested with \(BamHI/\lambda\)coI. Introduction of a single Cys residue by conversion of Lys at amino acid 75 to Cys to produce CaM(C75) was accomplished by polymerase chain reaction splicing by overlap extension (27). The presence of the desired mutation and the absence of polymerase chain reaction artifacts was confirmed by DNA sequencing. Cells expressing CaM were grown and lysed, and a soluble fraction was prepared as described previously (28). After dialysis against 50 mM Tris, pH 7.5, 0.2 mM EGTA, the soluble fraction was applied to a Macro-Prep Q 2.5 × 30-cm column (Bio-Rad), washed with the same buffer, and then eluted with a linear 0–500 mM KCl gradient. The appropriate fractions were pooled, made 1 mM in CaCl\(_2\), and loaded on a phenyl-Sepharose fast flow high-sub 2.5 cm column (Amersham). The column was washed with 50 mM Tris, pH 7.5, and 0.2 mM CaCl\(_2\) and then eluted with 50 mM Tris, pH 7.5, and 0.2 mM EGTA. If necessary, CaM was further purified by anion exchange chromatography using a semi-prep HPLC DEAE-5PW column ( Waters).

**Labeling of CaM(C75)** with IAE-DANS—IAE-DANS (Molecular Probes) was dissolved in N,N'-dimethylformamide at concentration of 100 mM and stored at 70°C. CaM(C75) at a concentration of 2–2.5 mg/ml was made 5 mM in dithiothreitol and then desalted into 50 mM MOPS, pH 7.0. Final protein concentrations were determined by absorbance spectroscopy using a molar extinction coefficient at 336 nm of 5700. Probe-to-protein ratios of bound probe was determined by absorbance spectroscopy using the BCA assay (Pierce) with CaM as a standard, and the amount of bound probe was determined by absorbance spectroscopy using a molar extinction coefficient at 336 nm of 5700. Probe-to-protein ratios were 0.9 and 1.0 for proteins labeled in the absence and presence of Ca\(^{2+}\)/CaM. This suggests that the minimum CaM-binding domain (residues 296–309) does not encompass all amino acids with the potential to interact with CaM.

**Characterization of Mutated and Labeled Calmodulin**—The cDNA for chicken calmodulin was mutagenized to introduce a single Cys residue for Lys at amino acid 75. A comparison of the crystal and NMR solution structures of free CaM (31) and CaM bound to a peptide derived from CaM-kinase II (22) indicated that the side chain of Lys 75 does not directly participate in peptide binding but would undergo a change in its environment upon formation of the peptide-CaM complex. Presumably, a probe bound to Cys at position 75 in CaM would not drastically influence the interaction of CaM with a target peptide or protein but would exhibit a change in fluorescence characteristics.
Fig. 1. CaM-kinase activation by recombinant and derivatized CaMs. The activity of recombinant \(\alpha\)-CaM-kinase (5 ng/assay) was determined in the presence of increasing concentrations of bovine brain CaM (●), recombinant bacterial-expressed CaM (rCaM; ○), or CaM(C75)\(_{\text{IAE}}\) (□). Serial dilutions of the CaM stocks were made in HEPES buffer, pH 7.5, containing 0.1 mg/ml BSA. CaM at the final concentrations indicated in the figure were added to reaction mixtures containing 25 mM HEPES, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.5 mM CaCl\(_2\), 10 mM MgCl\(_2\), 100 \(\mu\)M ATP, 5 \(\mu\)M CPT (PLRRTLSVAA) as substrate, and 0.1 mg/ml BSA. Reactions were terminated after 30 s, and activity was determined as described under “Experimental Procedures.” Activity is expressed as \(\mu\)mol/min/mg. The lines represent a fit of the experimental data to the Hill equation:

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A = A_{\text{max}}(1 + 10^{N\log(CaM_{50} - \log(CaM)))},
\]

where \(A\) is the activity at a given CaM concentration, \(A_{\text{max}}\) is the maximal activity, \(N\) is the Hill coefficient, and CaM\(_{50}\) is the concentration of CaM which yields 50% of maximal activity. The insets indicate the \(\log(CaM_{50})\) and Hill coefficients for each protein with percent errors derived from the computer fits.

Upon binding ligands, Fig. 1 shows that there is no discernible difference in the abilities of bovine brain CaM, recombinant CaM synthesized in bacteria, and CaM(C75)\(_{\text{IAE}}\) to activate CaM-kinase.

Effects of Different Peptides and CaM-kinase on Fluorescence of CaM(C75)\(_{\text{IAE}}\)—Two synthetic peptides were used in the current study (Fig. 2A). A crystal structure of the longer peptide, CKII(290–314), bound to CaM was reported by Meador et al. (22). The underlined residues in CKII(290–314) are those which were resolved in the crystal structure. The shorter peptide, CKII(296–312), is based on the minimal CaM-binding peptide (19). The C-terminal extension of Gly-Cys on the enzyme produced a second and larger increase in fluorescence intensity, although the absolute increase is smaller relative to either of the peptides. This increase in fluorescence intensity produced by the addition of CaM-kinase is similar to that reported by Meyer et al. (14). They further showed that the increase in fluorescence intensity was parallel by an increase in fluorescence anisotropy. Addition of 1 mM Mg\(^{2+}\)/ATP to the cuvette to allow autophosphorylation of the enzyme produced a second and larger increase in fluorescence, which is not seen when 1 mM Mg\(^{2+}\) is added to the cuvette (data not shown). The observed increase in fluorescence intensity is likely due to a combination of nucleotide binding and autophosphorylation of the enzyme and reached a stable plateau fluorescence increase within the few seconds required to open the fluorimeter, mix the sample manually, close the fluorimeter, and resume data collection. Similar to the two peptides, CaM appears to adopt distinct conformations when bound to the autophosphorylated and nonautophosphorylated enzyme. Interestingly, the final fluorescence intensity is equivalent for CaM(C75)\(_{\text{IAE}}\) bound to either CKII(290–314) or to autophosphorylated CaM-kinase. However, in interpreting this data, it is important to note that the similarities attained in intensity changes between the peptide-CaM(C75)\(_{\text{IAE}}\) complex and enzyme-CaM(C75)\(_{\text{IAE}}\) complex do not necessarily indicate that they adopt the same conformations.

Measurement of Calmodulin Off-rates using Steady-state and Stopped-flow Kinetics—The rate of dissociation of CaM(C75)\(_{\text{IAE}}\) from peptides or enzyme can be derived from the decrease in observed fluorescence as bound CaM(C75)\(_{\text{IAE}}\) is exchanged

Fig. 2. Peptide sequences and characterization of changes in emission spectra of CaM(C75)\(_{\text{IAE}}\) by binding Ca\(^{2+}\) and peptide. A, sequence of the two synthetic peptides used in the current study. In B, CaM(C75)\(_{\text{IAE}}\) (1 \(\mu\)g, 0.06 \(\mu\)g final concentration) was added to 1 ml of 25 mM MOPS, pH 7.0, 150 mM KCl, 0.1 mM EGTA, and 0.1 mg/ml BSA and an emission spectra obtained between 400 and 600 nm (–Ca\(^{2+}\)). CaCl\(_2\) (0.5 mM final concentration) was added and a second scan was obtained (+Ca\(^{2+}\)). Finally, an excess of CKII(296–312) was added (5 \(\mu\)g final concentration) and a third scan was taken (+Ca\(^{2+}\) + CKII(296–312)). The samples were excited at 345 nm at room temperature. The excitation and emission slit widths were 2 and 2 nm, respectively. Each trace shown is the average of three scans.
with excess unlabeled CaM. The fluorescence intensity after full exchange should approach the value for Ca\(^{2+}\)-bound CaM(C75)IAE. Fig. 4A shows the kinetics of dissociation of CaM(C75)IAE from CKII(296–312) or CaM-kinase which is not autophosphorylated. The data fit well to a single exponential equation (\(E = E_0 - k \cdot t\)) or from the dephosphorylated form of CaM-kinase (\(E = E_0 - k \cdot t\)) using a stopped-flow fluorimeter. CaM(C75)IAE (0.6 \(\mu\)M) in 25 mM MOPS, pH 7.0, 150 mM KCl, 0.1 mM EGTA, 0.1 mM MgCl\(_2\), and either CaM-kinase (0.6 \(\mu\)M) in 25 mM MOPS, pH 7.0, 150 mM KCl, 0.1 mM EGTA, 0.1 mM MgCl\(_2\), or and 0.5 mM CaCl\(_2\), and either CKII(290–314) or CaM-kinase (at approximately 0.6 \(\mu\)M) were rapidly mixed with CaM (30 \(\mu\)M) in the same buffer without peptide or enzyme at room temperature. Excitation was at 345 nm and emission was monitored using a 390-nm cut-off filter. Each curve represents the average of 5–6 exchange reactions. The solid lines and indicated rate constants were derived by fitting the experimental data to the single exponential equation \(E = E_0 - k \cdot t\) or from the phospho-form of CaM-kinase (\(E = E_0 - k \cdot t\)). The experimental design was exactly the same as described in the legend to Fig. 3, except that after addition of Ca\(^{2+}\) and peptide or CaM-kinase, 50-fold excess CaM was added to the cuvette, mixed manually, and data collected for the indicated times. A control to determine the extent of photobleaching was performed using CKII(296–312) without excess CaM (\(E = E_0 - k \cdot t\)). Excitation was at 345 nm and emission was monitored at 465 nm. Excitation and emission slit widths were 5 and 10 nm, respectively. Reactions were performed at room temperature.

**Fig. 3.** Comparison of fluorescence intensity changes of CaM(C75)IAE caused by addition of peptides or CaM-kinase. Steady-state fluorescence measurements were obtained as described in the legend to Fig. 2, except that the amount of added peptide (0.07 \(\mu\)M final concentration) or enzyme (3.5 \(\mu\)g, 0.07 \(\mu\)M final concentration) was sufficient to achieve a near maximal fluorescence increase without being in excess over CaM(C75)IAE (see under “Experimental Procedures”). Addition of Ca\(^{2+}\) produced similar increases in fluorescence in all three experiments. Addition of CKII(296–312) produced an increase in fluorescence intensity (\(E = E_0 - k \cdot t\)) or from the dephosphorylated form of CaM-kinase (\(E = E_0 - k \cdot t\)). Addition of CaM-kinase produced a small increase in fluorescence intensity that was greatly enhanced by the addition of 1 mM Mg\(^{2+}\)/ATP. The breaks in the time scale axis represent times when the instrument was paused (which varied from 2 to 6 s) to permit the addition and mixing of components. Subsequent addition of 1 mM EGTA resulted in a rapid decrease in fluorescence intensity that approached the initial value (data not shown). Excitation was at 345 nm and emission was monitored at 465 nm. Excitation and emission slit widths were 5 and 10 nm, respectively. Reactions were performed at room temperature.

**Fig. 4.** Measurement of CaM off-rates from peptides and CaMkinase using stopped-flow or steady-state kinetics. A, time course for CaM dissociation from CKII(296–312) (\(E = E_0 - k \cdot t\)) or from the dephosphorylated form of CaM-kinase (\(E = E_0 - k \cdot t\)) using a stopped-flow fluorimeter. CaM(C75)IAE (0.6 \(\mu\)M) in 25 mM MOPS, pH 7.0, 150 mM KCl, 0.1 mM EGTA, 0.1 mM MgCl\(_2\), and either CaM-kinase (0.6 \(\mu\)M) in 25 mM MOPS, pH 7.0, 150 mM KCl, 0.1 mM EGTA, 0.1 mM MgCl\(_2\), or and 0.5 mM CaCl\(_2\), and either CKII(290–314) or CaM-kinase (at approximately 0.6 \(\mu\)M) were rapidly mixed with CaM (30 \(\mu\)M) in the same buffer without peptide or enzyme at room temperature. Excitation was at 345 nm and emission was monitored using a 390-nm cut-off filter. Each curve represents the average of 5–6 exchange reactions. The solid lines and indicated rate constants were derived by fitting the experimental data to the single exponential equation \(E = E_0 - k \cdot t\) or from the phospho-form of CaM-kinase (\(E = E_0 - k \cdot t\)). The experimental design was exactly the same as described in the legend to Fig. 3, except that after addition of Ca\(^{2+}\) and peptide or CaM-kinase, 50-fold excess CaM was added to the cuvette, mixed manually, and data collected for the indicated times. A control to determine the extent of photobleaching was performed using CKII(296–312) without excess CaM (\(E = E_0 - k \cdot t\)). Excitation was at 345 nm and emission was monitored at 465 nm. Excitation and emission slit widths were 5 and 10 nm, respectively. The dotted line indicates the fluorescence intensity observed when 50-fold excess CaM is added prior to the addition of CKII(296–312). The indicated rate constants are estimates derived by fitting the experimental data to a single exponential equation in which the fluorescence intensity at maximal exchange is defined as the value indicated by the dotted line. performed at room temperature in the sustained presence of Mg\(^{2+}\)/ATP, it is likely that CaM-kinase underwent autophosphorylation at sites in addition to Thr\(^{286}\). We do not know how these subsequent autophosphorylation steps may influence the observed off-rates for CaM dissociation. Nevertheless, it is clear that CKII(290–314) kinetically mimics the trapped form of CaM binding to CaM-kinase.

**DISCUSSION**

We have shown that peptides which span different amino acids in the CaM-binding domain of CaM-kinase have greatly different rates of dissociation from CaM. CaM binding to peptide CKII(296–312) kinetically mimics binding to unphosphorylated CaM-kinase, while peptide CKII(290–314) mimics binding to the phosphorylated/trapped state of the enzyme. We
conclude that autophosphorylation of Thr286 in CaM-kinase is necessary for high affinity binding of CaM only within the context of the enzyme. Phosphorylation-dependent conformational changes in the CaM-binding region may allow formation of additional interactions with CaM that lead to a dramatic decrease in the rate of dissociation of CaM from CaM-kinase.

Phosphorylation-dependent conformational changes in CaM-kinase have been demonstrated previously by differential proteolysis. Kwiatkowski and King (23) showed that a site close to Thr286 becomes accessible to proteolysis only when Thr286 is autophosphorylated, indicating exposure of this area. Subsequently, Yamagata et al. (24) showed that limited chymotrypsin treatment cleaved CaM-kinase at Phe283 in the absence of phosphorylation and at Ile272 when autophosphorylated. Together, these results indicate that the region just N-terminal to the CaM-binding domain of CaM-kinase undergoes a conformational change of sufficient magnitude to expose the region to proteolytic attack following autophosphorylation of Thr286. Because the peptide mimics the autophosphorylated enzyme, we predict that this conformational change allows new contacts to form between CaM and CaM-kinase following autophosphorylation leading to the high affinity CaM-binding state.

Our results suggest that the crystal structure resolved for the complex of CaM and CKII(290–314) represents high affinity binding of CaM to CaM-kinase. In this structure, only amino acids 293–310 form stable interactions with CaM (see underlined residues in Fig. 2A) and are seen as an α-helical rod that is enveloped by CaM (22). Amino acids 290–292 and 311–314 are not resolved in the crystal structure, presumably because they do not interact with CaM and are free to adopt random conformations. Thus, amino acids included in the CaM-binding region as defined by the crystal structure, but which are absent in the minimal CaM-binding peptide, are Phe283, Asn284, and Ala285. It seems most probable that these residues are responsible for the difference in dissociation rates of the two peptides from CaM.

If one assumes similar rates of association of CaM from CKII(296–312) and CKII(290–314), as is seen for binding CaM to phospho- and dephospho-CaM-kinase (14), then the difference in free energy between complexes of CaM and the two peptides would be about 7 kcal/mol. Thus, the amino acids 293–310 form stable interactions with CaM (see underlined residues in Fig. 2A) and are seen as an α-helical rod that is enveloped by CaM (22). Amino acids 290–292 and 311–314 are not resolved in the crystal structure, presumably because they do not interact with CaM and are free to adopt random conformations. Thus, amino acids included in the CaM-binding region as defined by the crystal structure, but which are absent in the minimal CaM-binding peptide, are Phe283, Asn284, and Ala285. It seems most probable that these residues are responsible for the difference in dissociation rates of the two peptides from CaM.

The results described in the present study also suggest an important potential limitation on the reversibility of the CaM trapping process. Once CaM-kinase is autophosphorylated and converted to the CaM-trapped state, dephosphorylation may not completely return the off-rate for CaM to the basal state. This hypothesis is based on the observation that the synthetic peptide CKII(290–314) kinetically mimics the trapped state and has no associated phosphate group. Once CaM is trapped, autophosphorylation of Thr286 may no longer be essential. This is not meant to indicate that dephosphorylation of Thr286 would have no effect on CaM-binding affinity. A reasonable prediction would be that an intermediate affinity may be obtained between the fully trapped (autophosphorylated) and untrapped (dephosphorylated) form of the enzyme. In either case, once established in the trapped conformation, the dominant mechanism for CaM dissociation from the enzyme would be to decrease Ca2⁺ concentrations. Future experiments will evaluate whether dephosphorylation of autophosphorylated CaM-kinase causes alterations in CaM off-rate of the enzyme in the presence of maintained Ca2⁺ levels.

In summary, the data presented here, together with an analysis of the crystal structure of CaM bound to CKII(290–314) and previous studies using peptides and limited proteolysis, provide a model that can be tested for the way in which CaM-kinase transitions from the low to high affinity CaM binding, which involves the following events: 1) CaM binds to two adjacent subunits in CaM-kinase holoenzymes, which likely involves amino acids 296–310 of each subunit; 2) intersubunit autophosphorylation of Thr286 occurs following Mg2+/ATP binding; 3) a conformational change is induced in the CaM-binding region; and 4) stabilizing contacts between CaM and CaM-kinase are established, which may include hydrophobic interactions involving Phe283 and Ala285 and salt bridges involving Arg-Arg-Lys286.

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