Molecular Characterization of Calmodulin Trapping by Calcium/Calmodulin-dependent Protein Kinase II*

Sheela I. Singla‡, Andy Hudmon‡, Jonathan M. Goldberg§, Janet L. Smith§¶, and Howard Schulman¶¶

From the ‡Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5125 and the ¶Boston Biomedical Research Institute, Watertown, Massachusetts 02472

Autophosphorylation of α-Ca²⁺/calcmodulin-dependent protein kinase II (CaM kinase II) at Thr²⁹⁶ results in calmodulin (CaM) trapping, a >10,000-fold decrease in the dissociation rate of CaM from the enzyme. Here we present the first site-directed mutagenesis study on the dissociation of the high affinity complex between CaM and full-length CaM kinase II. We measured dissociation kinetics of CaM and CaM kinase II proteins by using fluorescently modified CaM that is sensitive to binding to target proteins. In low [Ca²⁺], the phosphorylated mutant kinase P293A and the CaM mutant E120A/M124A exhibited deficient trapping compared with wild-type. In high [Ca²⁺], the CaM mutations E120A, M124A, and E120A/M124A and the CaM kinase II mutations F293A, F293E, N294A, N294P, and R297E increased dissociation rate constants by factors ranging from 2.3 to 116. We have also identified residues in CaM and CaM kinase II that interact in the trapped state by mutant cycle-based analysis, which suggests that interactions between Phe²⁹³ in the kinase and Glu¹²⁰ and Met¹²⁴ in CaM specifically stabilize the trapped CaM-CaM kinase II complex. Our studies further show that Phe²⁹³ and Asn²⁹⁴ in CaM kinase II play dual roles, because they likely destabilize the low affinity state of CaM complexed to unphosphorylated kinase but stabilize the trapped state of CaM bound to phosphorylated kinase.

Ca²⁺/calcmodulin-dependent protein kinase II (CaM kinase II) is a multifunctional Ser/Thr protein kinase that mediates numerous cellular effects of calcium signals (for reviews, see Refs. 1 and 2). The neuronal form of CaM kinase II is composed primarily of α (54 kDa) and β (60 kDa) subunits and appears to exist as a mixed holoenzyme (3) of 12 subunits (4). The enzyme is maintained in an inactive state by an autoinhibitory regulatory domain, and binding of Ca²⁺/CaM relieves the autoinhibitory effect to increase both kinase autophosphorylation and phosphorylation of exogenous substrates. The primary site of autophosphorylation, Thr²⁹⁶, is within the regulatory domain, and phosphorylation of this site proceeds by an intraholoenzyme, intersubunit mechanism (5–7).

Autophosphorylation of Thr²⁹⁶ is necessary and sufficient for generation of an autonomous enzyme (1), which has partial activity in the absence of Ca²⁺/CaM. In addition, autophosphorylation of Thr²⁹⁶ slows the dissociation of CaM from the kinase by a factor of about 13,000 (8), a phenomenon termed CaM trapping (9). Trapping likely accounts for the ability of CaM kinase II to decode the frequency of Ca²⁺ oscillations into distinct levels of kinase activity in vitro and may play a similar role in vivo (10). In addition, trapped CaM prevents autophosphorylation of the CaM binding domain at Thr³⁰⁵ and Thr³⁰⁶ (11). Phosphorylation at these sites blocks re-binding of CaM and thereby facilitates dissociation of the holoenzyme from anchoring protein(s) at synaptic sites (12). Finally, because there appears to be an excess of CaM-binding proteins over free CaM in most types of cells (13, 14), the high concentration of CaM kinase II in neuronal compartments (15) may allow this enzyme to act as a regulatable CaM sink to limit free CaM levels. Thus, an understanding of the molecular mechanism of trapping would be a significant aid in attempting to elucidate the biological roles of CaM trapping by CaM kinase II.

CaM has been crystallized in a complex with a peptide corresponding to residues 290–314 from CaM kinase II (16). We wished to understand how CaM interacts with this target helix in the context of intact CaM kinase II, and whether it makes additional interactions, either favorable or unfavorable, with the full-length protein. We also sought to determine the energetic importance of the interactions, because proximity in space does not in general correlate with thermodynamic importance (17). Waxham et al. (18) showed that peptides truncated at the N-terminal part of the target helix, which are missing residues Phe²⁹³-Asn²⁹⁴-Ala²⁹⁵, have low affinity for CaM, and that the three basic residues Arg²⁸⁹-Arg²⁹⁷-Lys²⁹⁸ make a much larger contribution to binding CaM when peptides are extended to include Phe²⁹³-Asn²⁹⁴-Ala²⁹⁵. They proposed that autophosphorylation might promote trapping by making Phe²⁹³-Asn²⁹⁴-Ala²⁹⁵ available to interact with CaM, which in turn allows Arg²⁸⁹-Arg²⁹⁷-Lys²⁹⁸ to make favorable interactions with CaM. Any model that proposes that this or other portions of the target helix are inaccessible in intact CaM kinase II are most readily tested by mutating the key residues in the context of the full-length protein. In addition, a significant portion of the binding energy may come from interactions between the surface of CaM and CaM kinase II, particularly the phosphate moiety of Thr²⁹⁶. Interactions between polar and charged groups on the surface of CaM and regions of target enzymes.
distinct from the CaM binding domain are thought to be important for complete activation of some protein kinases (19), and in the crystal structure of CaM bound to *Anthrax* adenyl cyclase, extensive interactions between the surface of calmodulin and the target protein are observed.2

We have tested these models for trapping by mutating candidate residues in the context of full-length CaM kinase II and CaM. We find that mutation of Phe293, Asn294, or Arg297 decreases CaM binding to autophosphorylated but not unphosphorylated kinase. We also show that mutating the CaM residues Glu120 and Met124 affects binding to only autophosphorylated kinase, and we have used mutant cycle analysis, which quantifies the degree of interaction between residues, to show that these mutations are coupled to mutations of Phe293.

The proposed biological roles for trapping rely on the presence of a large difference in the affinity of CaM kinase II for CaM before and after autophosphorylation. An unexpected finding of our study is that the F293A and N294A mutations not only weaken binding to CaM when the kinase is autophosphorylated but also strengthen CaM binding to unphosphorylated kinase, presumably because these mutations also partially disrupt autoinhibition. In these mutants, $k_{on}$ only decreases 160- and 330-fold, respectively, after autophosphorylation, rather than the 23,000-fold decrease observed for wild-type. These mutants are thus excellent candidates for in vivo studies aimed at further elucidating the biological role of trapping.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification of Calmodulin**—The CaM expression plasmid pCR2 was a gift from Drs. Carol Rohl and Rachel Klevit. This construct contains the sea urchin CaM cDNA (identical in predicted amino acid sequence to vertebrate CaM) in the *Escherichia coli* expression vector pET23d (Novagen). All mutagenesis was performed with the Transformer site-directed mutagenesis kit (CLONTECH), and all mutations were verified by DNA sequencing. CaM was expressed in the bacterial strain BL21(DE3) (Novagen). Liquid cultures were grown at 37 °C to an $OD_{600}$ of 0.8–1.0 and then induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside. After 4–6 h, cells were harvested at 8670 $\times$ g for 7 min and resuspended in $\nu$/o the culture volume of buffer. The resuspended pellets were stored at –80 °C.

Two methods were used for purification of the recombinant CaMs. All steps were performed at room temperature, unless otherwise indicated. The first method was essentially as described (20), with the following modifications: from 500 ml of culture that had been resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mg/ml lysozyme) were thawed, and the bacterial DNA was digested by a 15-min incubation in lysis buffer plus 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 2.5 mM MgCl2 DNase I. The solution was then clarified by centrifugation at 12,000 $\times$ g, 2.5 mg/ml DNase I. The solution was then clarified by centrifugation at 12,000 $\times$ g, 30-min incubation of EGTA needed to lower free Ca2+ concentration of 15–50 mM. In the second method, the pooled fractions were desalted with 50 mM MOPS, pH 7.0, 1 M NaCl, 1 mM EDTA, 1× complete protease inhibitors mixture (Roche Molecular Biochemicals). The resuspended pellets were lysed by Dounce homogenization and sonication and clarified by centrifugation at 100,000 $\times$ g for 30 min. The supernatant was then loaded onto a 5-ml phenyl-Sepharose column (Amersham Pharmacia Biotech) and further desalted by 3–4 cycles of concentration and dilution in 50 mM MOPS, pH 7.0 (ultra-free spin column, molecular weight 5000 cutoff, Millipore). Yield was 50–75 mg of protein from 500 ml of bacterial culture, and CaM was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. Purified CaM preparations were stored at –20 or –80 °C. CaM preparations were verified by Western analysis using an anti-Thr286 antibody or by measurement of autonomous activity as able to activate the kinase for autophosphorylation of Thr286.

**Labeling of CaM (K75C)** with N-Bromoacetetyl-N-5-sulfo-1-naphthylethylene-diamine—All CaM mutants were generated in a K75C background for labeling purposes. Labeling was done as previously described (8), except that urea was not included. Excess uncoupled fluorophore was removed by desalting (Hi-Trap desalting column, Amersham Pharmacia Biotech) and further desalted by 3–4 cycles of concentration and dilution in 50 mM MOPS, pH 7.0 (ultra-free spin column, molecular weight 5000 cutoff). Concentration and probe-to-protein ratios (0.8 to 1) were determined as described (8).

**Mutagenesis, Expression, and Purification of CaM Kinase II**—The rat a-CaM kinase II cDNA in the vector pBakPak9 (CLONTECH) and virus containing the wild-type a-CaM kinase II cDNA were gifts from Dr. Neal Waxham (8). The kinase II gene was subcloned from pBak-Pak9 into the *Bam*HI and EcoRI sites of the insect cell shuttle vector pFastBac1 (Life Technologies, Inc.). Mutagenesis of the CaM kinase II cDNA (Transformer site-directed mutagenesis kit, CLONTECH) was carried out either directly in pFastBac1 or in the pSRu vector (21), followed by subcloning of the mutated fragment into pFastBac1. Mutant and wild-type kinases were expressed using the baculovirus-SF21 cell expression system (Life Technologies, Inc.). Viruses containing mutant forms of the CaM kinase II cDNA were produced in monolayer or suspension culture of SF21 cells as described by the manufacturer. Proteins were expressed in SF21 suspension cultures for 60–72 h, at which point, cells were harvested, and pellets were stored at –80 °C until purification.

**For purification**, cell pellets were resuspended in 50 mM PIPES, pH 7.0, 2.5% or 5% betaine, 1 mM EGTA, 1 mM EDTA, 1× complete protease inhibitors mixture (Roche Molecular Biochemicals). The resuspended pellets were lysed by Dounce homogenization and sonication and clarified by centrifugation at 100,000 $\times$ g for 30 min. The supernatant was then loaded onto a 5-ml phenyl-Sepharose column (P11 cation exchange resin, Whatman) equilibrated in 50 mM PIPES, pH 7.0, 100 mM NaCl, 1 mM EDTA, 1× complete protease inhibitors mixture. The CaM kinase II was eluted from the phenyl-Sepharose column in a 30-ml wash plus 100 to 0.5 M NaCl, 10 mM MgCl2, 10 mM CaCl2, and 10 mM EGTA. The appropriate fractions were pooled, 2.5 mM CaCl2 was added to the pool, and then the pool was loaded onto a 5–10 ml CaM-Sepharose column (Amersham Pharmacia Biotech) equilibrated in 40 mM HEPES, pH 7.3, 2 mM CaCl2, 10% glycerol, 0.1 mM NaCl. The flow-through was reloaded, and the column was washed with loading buffer plus 0.5–2 mM NaCl. Kinase was then eluted with 40 mM HEPES, pH 7.3, 10% glycerol, 0.5 mM CaCl2, 2 mM EDTA, and protein was detected by the Bradford method. The pooled CaM-Sepharose eluate was made a total of 30% glycerol and then snap-frozen in a dry ice/ethanol bath and stored at –80 °C. Final concentrations were determined by Bradford Assay with bovine serum albumin as the standard. Specific activities of the kinases ranged from 15–50 pmol/min/mg of protein with 50 mM ATP, 200–500 μM [γ-32P]ATP (120–300 Ci/mmol, 500 μM CaCl2, 150 mM RCl, 10 mM MgCl2, and 2 mM CaCl2.

**Determination of Free CaCl2**—Free calcium concentrations for CaM-kinase fluorescence experiments were determined by a fluorometric assay using fluo-3 (Molecular Probes). A standard curve was generated as described by the manufacturer, and then the appropriate concentration of EGTA needed to lower free [CaCl2] to 200–500 μM in our CaM-kinase fluorescence conditions was determined experimentally.

**Determination of Kinetic Constants**—Slow off-rates were measured in an Amino-Bowan-2 fluorometer. All measurements were performed at 30 °C, and samples were continually mixed with a stir-bar. Excitation was at 345 nm, and the emission monochromator was set to 465 nm. Band-passes were 4 nm (excitation and emission). Each high [CaCl2] experiment was done in 1.5 ml of 50 mM MOPS, pH 7.0, 150 mM CaCl2, 0.5 mM CaCl2, followed by elution with 50 mM Tris, pH 7.5, 1 mM NaCl, 1 mM EDTA, 150 mM CaCl2, Bio-Rad assay.

---

2 A. Bohm, personal communication.
either a 50–75-fold molar excess of unlabeled Ca²⁺ is from the crystal structure of the CaM-peptide complex (16). For each peptide residue that is mutated, all of the CaM residues that contact it in the crystal structure are indicated by lines to the appropriate residue in CaM below it. An arrow to the substituted amino acid indicates each mutation, and double mutants are indicated by an underline. For residues that were mutated to more than one amino acid, the alternate substitution is shown above the first substitution. Arg¹⁰⁶ and Arg¹²⁶ in CaM do not contact the peptide, and Leu²⁹⁰, Lys²⁹¹, and Lys²⁹² in the peptide were not ordered in the crystal structure.

We sought to identify amino acid residues in full-length CaM kinase II and their binding partners in CaM that contribute to trapping (high affinity binding state) but are not important for CaM binding to unphosphorylated CaM kinase II (low affinity binding state). A further objective was to characterize the thermodynamic importance of these residues. These goals were facilitated by a fluorescent CaM, CaM (K75C)ΔAR, which activates CaM kinase II with identical concentration dependence as wild-type unlabeled CaM (8). Using the CaM (K75C) background (hereafter identified solely by mutations additional to K75C), further mutations were constructed to determine the kinetics of dissociation from CaM kinase II in the autophosphorylated and unphosphorylated states.

In the x-ray crystal structure of CaM bound to the peptide CaM kinase II 290–314 (16), Phe²⁹³ interacts with Glu¹²⁰ and Met¹²⁴ in CaM, and Waxham et al. (18) have proposed that Asn²⁹⁴ and Arg²⁹⁸ in the kinase and interactions between CaM and Phe²⁹³ are critical for trapping. We have studied mutants with substitutions at each of these positions, both in order to test this model and to determine the thermodynamic importance of these interactions, which cannot be reliably deduced from the crystal structure (17). The mutants studied are summarized in Fig. 1.

Effects of Mutations on Trapping in Low [Ca²⁺]—In the initial characterization of CaM trapping, Meyer et al. (9) showed that autophosphorylation induces a 20-fold to more than 1000-fold decrease in the dissociation rate of CaM from CaM kinase II, depending on the Ca²⁺ concentration. This study further showed that the increased affinity of phospho-CaM kinase II for CaM is almost fully the result of changes in the off-rate. Therefore, we measured the kinetics of dissociation of CaM and CaM kinase II with mutations hypothesized to selectively affect trapping. Phosphorylated CaM kinase II (F293A) releases CaMΔAR considerably faster than does phosphorylated wild-type kinase, whereas low affinity binding to unphosphorylated kinase is only slightly affected (Fig. 2). CaMΔAR (E120A/M124A) also dissociates from phospho-CaM kinase II considerably faster than does wild-type CaMΔAR, whereas binding to unphosphorylated kinase changes only slightly (Fig. 2).

The fluorescence traces in Fig. 2 have two kinetic components; this is most likely the result of a decrease in fluorescence when one or more Ca²⁺ ions dissociate, followed by a decrease when apoCaM dissociates from the kinase. Based on dissociation of Ca²⁺ and calmodulin from other enzymes and peptides, it is likely that the faster component corresponds to dissociation of Ca²⁺ from its low affinity sites on CaM and the slower component corresponds to dissociation of Ca²⁺ from its high affinity sites, followed by rapid dissociation of apo-CaM (22, 23). The rate constants were as follows: for unphosphorylated enzyme, WT CaM/WT kinase, 13 and 3.6 s⁻¹; F293A kinase/WT CaM, 15 and 3 s⁻¹; WT kinase/E120A/M124A CaM, 10 s⁻¹. Thus, the mutations have little effect on dissociation from unphosphorylated enzyme. For phosphorylated enzyme, the rate constants were increased 5–10-fold for the mutants, and were as follows: WT kinase/WT CaM, 0.04 and 0.01 s⁻¹; F293A kinase/WT CaM, 0.39 and 0.05 s⁻¹; WT kinase/E120A/M124A CaM, 0.15 s⁻¹. The E120A/M124A CaM/WT kinase complex exhibited monoexponential dissociation kinetics, implying that the four Ca²⁺ ions dissociate in bulk, as was observed for the CaM-myosin light chain kinase complex (24). These results implicate Phe²⁹³ in CaM kinase II and Glu¹²⁰ and Met¹²⁴ in CaM as important for high affinity binding to phosphorylated kinase and relatively unimportant for low affinity binding to unphosphorylated kinase.

Effects of Mutations on Trapping in High [Ca²⁺]—Fig. 3 shows that autophosphorylation of CaM kinase II results in...
trapping of CaM in high [Ca\(^{2+}\)]. The measured CaM\(_{IAE}\) dissociation rate constant for unphosphorylated CaM kinase II, 2.2 s\(^{-1}\) (Table I) agrees well with previous measurements of 2 s\(^{-1}\) (9) and 1.1 s\(^{-1}\) (8). Similarly, our determination of \(k_{off}\) of CaM for autophosphorylated CaM kinase II, 9.5 \(\times 10^{-2}\) s\(^{-1}\), is comparable to a previously determined value (8.7 \(\times 10^{-5}\) s\(^{-1}\)) (8). Thus, autophosphorylation results in a 13,000-fold (8) to 600-fold (9) increase in \(k_{off}\) values in high [Ca\(^{2+}\)].

Also consistent with the results in low [Ca\(^{2+}\)], CaM mutants E120A, M124A, and E120A/M124A all dissociated from phosphorylated CaM kinase II faster than wild-type CaM\(_{IAE}\) (Fig. 4A; Table I). These results indicate that the Glu\(^{120}\) and Met\(^{124}\) side chains preferentially stabilize the trapped state. This mutation N294A also results in deficient trapping, as CaM\(_{IAE}\) was exchanged with a dissociation rate constant 5.2 times lower than that of the wild-type kinase (Fig. 3; Table I). In the crystal structure, this residue does not make any contacts with CaM (16), but it N-caps the target a-helix by forming a hydrogen bond with the backbone amide of Arg\(^{297}\).

Furthermore, the kinase mutations F293A and N294A have the interesting property of enhancing binding to unphosphorylated kinase and weakening binding to phosphorylated kinase (Figs. 3 and 4). These results suggest that Phe\(^{293}\) and Asn\(^{294}\) play dual roles in the trapping process, because the phosphorylation state of Thr\(^{286}\) determines whether they stabilize or destabilize the CaM-bound form of the enzyme.

Because peptide studies indicate that the basic residues in the region of Arg\(^{297}\) in the kinase make a much larger contribution to CaM binding in peptides that contain Phe\(^{293}\) and Asn\(^{294}\) (18), we tested the kinase mutant R297E. This mutant did exhibit deficient trapping (Figs. 3 and 4B), whereas low affinity binding was very slightly enhanced, indicating that the Glu\(^{120}\) and Met\(^{124}\) side chains preferentially stabilize the trapped state. The kinase mutation N294A also results in deficient trapping, as CaM\(_{IAE}\) was exchanged with a dissociation rate constant 5.2 times lower than that of the wild-type kinase (Fig. 3; Table I). In the crystal structure, this residue does not make any contacts with CaM (16), but it N-caps the target a-helix by forming a hydrogen bond with the backbone amide of Arg\(^{297}\).
CaM Trapping by CaM Kinase II

**Table I**

Summary of effects of alanine mutations in CaM and CaM kinase II on off-rates in high [Ca\(^{2+}\)]

<table>
<thead>
<tr>
<th>CaM kinase II</th>
<th>Calmodulin</th>
<th>(k_{\text{off}}) (\times 10^4) (phosphokinase)</th>
<th>(k_{\text{off}}) (\times 10^4) (non-phosphokinase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Wild-type</td>
<td>0.95 ± 0.05 (^a)</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>L105A</td>
<td>0.50 ± 0.09</td>
<td>ND</td>
</tr>
<tr>
<td>Wild-type</td>
<td>R106A/R126A</td>
<td>69 ± 0.23</td>
<td>ND</td>
</tr>
<tr>
<td>Wild-type</td>
<td>E120A</td>
<td>2.2 ± 0.3</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>Wild-type</td>
<td>M124A</td>
<td>3.2 ± 0.3 (^a)</td>
<td>4.0 ± 1</td>
</tr>
<tr>
<td>Wild-type</td>
<td>E120A/M124A</td>
<td>16 ± 1</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>F293A</td>
<td>Wild-type</td>
<td>8.6 ± 1</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>F293A</td>
<td>E120A</td>
<td>14.3 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>F293A</td>
<td>M124A</td>
<td>14.3 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>N294A</td>
<td>Wild-type</td>
<td>5.2 ± 0.7</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>N294A</td>
<td>E120A</td>
<td>10.9 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>N294A</td>
<td>M124A</td>
<td>12.7 ± 0.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Best fit by the double exponential equation.

If the mutated residues behave independently of each other, \(\Omega\) will be equal to 1. If the mutated residues interact with each other and the mutations affect that interaction, \(\Omega\) will deviate in either direction from 1. In order to be consistent with the literature and so that comparisons can be more readily made, we have inverted \(\Omega\) (expressed as \(\Omega^{-1}\)) if less than 1.

In order to assess whether and to what extent Phe\(^{293}\) in autophosphorylated CaM kinase II interacts with Glu\(^{120}\) and Met\(^{124}\) in CaM, we measured dissociation rates of CaM\(_{\text{IAE}}\) (E120A) and CaM\(_{\text{IAK}}\) (M124A) from either phospho-CaM kinase II (WT) or from phospho-CaM kinase II (F293A). We found that both CaM mutations E120A and M124A are coupled to F293A (\(\Omega^{-1} = 1.4 ± 0.3\) and 2.0 ± 0.3, respectively), implying that both Glu\(^{120}\) and Met\(^{124}\) in CaM interact with Phe\(^{293}\) in phosphorylated CaM kinase II. Fig. 5 shows a comparison between the observed off-rate of the mutant kinase/mutant CaM complex (observed, black bars) and the off-rate that would be expected for this complex if the effects of the individual mutations were additive, i.e. acting independently of each other (additive, white bars).

The magnitude of \(\Omega\) reflects the degree to which mutations affect an interaction as well as the strength of the interaction itself (25). The mutations produced here resulted in relatively small \(\Omega\) values, compared with those in Hidalgo and MacKinnon (25). One might predict that the inherent flexibility of CaM and the plasticity of its extensive hydrophobic interactions with varying targets (16, 19, 33–35) contributed to the differing results, because the scorpion toxin-potassium channel system studied in Ref. 25 is characterized by a rigid, inflexible binding interface that is dominated by electrostatic interactions. Also, our choice of alanine mutations may have contributed to the observed \(\Omega\) magnitudes; therefore, we created the potentially more disruptive CaM kinase II mutant F293E and tested coupling with CaM (E120A) and CaM (M124A). This F293E mutation did result in a lower \(\Omega\) value \((\Omega^{-1} = 1.9 ± 0.8\) and 5.2 ± 1.8, respectively; Fig. 5), thus supporting the conclusion that Glu\(^{120}\) and Met\(^{124}\) in CaM interact with Phe\(^{293}\) in phospho-CaM kinase II.

The kinase mutant N294A was then tested for coupling with the CaM mutations E120A and M124A, yielding \(\Omega^{-1} = 1.1 ± 0.3\) and 1.3 ± 0.4, respectively. These results suggest that Asn\(^{294}\) is not coupled to Glu\(^{120}\) but may be weakly coupled to Met\(^{124}\). A potentially more disruptive mutation at this position, N294P, was tested, and the resulting \(\Omega\) values (\(\Omega^{-1} = 1.0 ± 0.3\) and 1.2 ± 0.3, respectively; Fig. 5) indicate no coupling between Asn\(^{294}\) and Glu\(^{120}\) and Met\(^{124}\). A proline at this position may be less efficient in substituting for the native asparagine than alanine in the context of CaM mutations at 120 and 124, thus yielding \(\Omega\) values closer to unity. This result suggests that Asn\(^{294}\) in the kinase participates in stabilization of the trapped state independently of an interaction with Glu\(^{120}\) and Met\(^{124}\).

Having established that Glu\(^{120}\) and Met\(^{124}\) in CaM participate in trapping by interacting with Phe\(^{293}\) in CaM kinase II (Fig. 5) and that the kinase mutant R297E results in deficient trapping (Figs. 3 and 4B), we tested coupling between them by measuring the off-rate of CaM (E120A/M124A) from phospho-CaM kinase II (R297E). There is minimal coupling between the mutations E120A and M124A in CaM and the nonconservative R297E mutation in phosphorylated CaM kinase II, as can be seen by comparing kinase (R297E)-CaM (E120A/M124A) with kinase (F293E)-CaM (E120A) and kinase (F293E)-CaM (M124A) in Fig. 5. This implies that Arg\(^{297}\) stabilizes the CaM-bound state independently of interactions with Glu\(^{120}\) and Met\(^{124}\). This combination results in a 116-fold increase in the off-rate of CaM (Fig. 3; Table II), the greatest single effect observed in this study.
The effects of CaM kinase II mutations on CaM trapping and low affinity binding in high Ca$^{2+}$. A shows the effects of CaM mutations on $k_{\text{off}}$ from unphosphorylated (white squares) and phosphorylated (black circles) CaM kinase II, and B shows the effects of CaM kinase II mutations on $k_{\text{off}}$ for wild-type CaM from unphosphorylated (white squares) and phosphorylated (black circles) kinase. $k_{\text{off}}$ is presented on a log scale so that measurements for phosphorylated and unphosphorylated kinase may be readily viewed on the same plot. The dotted lines indicate the values for the dissociation of wild-type CaM from wild-type kinase. Experiments were as described in Fig. 3.

**Table II**

Summary of effects of nonconservative mutations on CaM kinase II on off-rates in high (Ca$^{2+}$)

All experiments were done by conventional fluorimetry. Exchange of Ca$^{2+}$/CaM from autoinhibited CaM kinase II was initiated by addition of a 50–75-fold molar excess of unlabeled CaM. First-order rate constants, $k_{\text{off}} \pm$ S.D., are the average values from fitting the data from at least three independent experiments to the exponential equation $F(t) = F_0 \times 10^{-k_{\text{off}} \times t}$.

<table>
<thead>
<tr>
<th>Phospho-CaM kinase II</th>
<th>Calmodulin</th>
<th>$k_{\text{off}} \times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K291E</td>
<td>Wild-type</td>
<td>0.934 ± 0.003</td>
</tr>
<tr>
<td>F293E</td>
<td>Wild-type</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>F293E</td>
<td>E120A</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>F293E</td>
<td>M124A</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>N294P</td>
<td>Wild-type</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>N294P</td>
<td>E120A</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>N294P</td>
<td>M124A</td>
<td>9.4 ± 1.8</td>
</tr>
<tr>
<td>R297E</td>
<td>Wild-type</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>R297E</td>
<td>E120A/M124A</td>
<td>110 ± 4</td>
</tr>
</tbody>
</table>

**Discussion**

In this study, we have described amino acid interactions that are specifically critical for CaM binding to autoinhibited CaM kinase II, i.e. the trapped state. The results indicate that Phe293 in the phosho-kinase interacts with Met124 and Glu120 in CaM, and that Asn294 stabilizes the trapped form without interacting with Met124 and Glu120, and that Arg297 in CaM kinase II also provides a portion of the binding energy that stabilizes the trapped state.

The x-ray crystal structure of autoinhibited Ca$^{2+}$/calmodulin-dependent protein kinase I (CaM kinase I) suggests a mechanism by which Phe293 and Asn294 may be prevented from interfering with CaM until after autophosphorylation of Thr286. In this structure, Val290 and Ile294 in the αR1 CaM-binding helix of CaM kinase I are buried in a hydrophobic channel in the catalytic core of the enzyme (36). The corresponding residues in CaM kinase II, Thr296 and Leu298, have been proposed to pack into a similar hydrophobic groove in the autoinhibited kinase (37). CaM binding to the unphosphorylated kinase may only partially disrupt this extensive packing, so that autophosphorylation of Thr286 would be necessary to fully displace the region αR1-terminus to Phe293 and Asn294. Full dissociation of the autoinhibitory domain from a hydrophobic and acidic environment by repulsion of phospho-Thr286 (37) may then result in further accessibility of Phe293 and Asn294 so that maximal binding interactions with CaM can be realized.

A schematic model of the effects of phosphorylation of Thr286 on Ca$^{2+}$/CaM binding to CaM kinase II is shown in Fig. 6. The autoinhibitory domain of an unphosphorylated kinase makes a number of contacts with the catalytic core (Fig. 6A) that are disrupted by binding of Ca$^{2+}$/CaM (Fig. 6B), leading to maximal activation of the enzyme. However, the full target sequence is unavailable for CaM until Thr286 is phosphorylated (Fig. 6C). This suggests that some interactions between the autoinhibitory domain and the catalytic core are retained in the activated enzyme prior to autophosphorylation and effectively compete with CaM interaction. The E120A/M124A mutation of CaM kinase II increases $k_{\text{off}}$ in the context of phosphorylated kinase and has little effect on dissociation from unphosphorylated enzyme, suggesting that this segment of CaM interacts only with phosphorylated CaM kinase II. Moreover, mutations of Phe293 increase $k_{\text{off}}$ in the context of phosphorylated enzyme and are coupled with the E120A and M124A mutations of CaM, indicating that interactions between Phe293 of CaM kinase II and CaM are.
CaM binding and phosphorylation are represented by nase, respectively. Noncovalent interactions that change as a result of relative strengths of interactions are inferred from k

Thr286 of CaM kinase II on Ca2

display minimal coupling with Glu120 and Met124 on CaM (Fig. 6, B). The importance of Phe 293, Asn 294, and Arg 297 was first suggested based on peptide studies (18), and the current study supports this suggestion in the physiologically relevant case of full-length CaM kinase II.

Phe293 and Asn294 contribute to the autoinhibition of kinase (37, 38), raising the question of how the model in Fig. 6 accounts for enzymatic activity in the absence of autophosphorylation. It is possible that, as shown in Fig. 6B, the nature of the interaction with the catalytic core changes upon CaM binding, such that the contacts no longer inhibit the enzyme but are able to constrain Phe293 and Asn294 so that they are unavailable for CaM. A relatively small conformational change at this interface may be sufficient to confer a large increase in enzymatic activity as the binding energy is propagated through the residues that contact Phe293 and Asn294 to the active site (17).

Previous studies have suggested the importance of Met124 in CaM in activation of CaM kinase II and other protein kinases (39, 40). For CaM kinase II, the M124Q mutation increases KcA 25-fold (40). The present study is consistent with these observations, because the requirement that CaM bind to two subunits of the holoenzyme for autophosphorylation to occur (5, 6) would be more difficult to achieve with the trapping-deficient mutant CaM (M124A) and would therefore be predicted to cause an increase in the KcA for autophosphorylation. Previous studies have also proposed that the flexible side chains of methionine and glutamate allow CaM to adjust its interactions to optimize binding with variable target sequences (41). Our study supports this suggestion, as the intrinsic conformational flexibility of the Met124 and Glu120 side chains likely permits additional contacts to be formed with residues on the N-terminal side of the core CaM binding domain after autophosphorylation of CaM kinase II. Moreover, the flexible central helix of CaM was observed to be important for target-specific positioning of the two lobes in binding peptides corresponding to the different length CaM binding domains of smooth muscle myosin light chain kinase and CaM kinase II (16, 33) and may serve similarly as an expansion joint when autophosphorylation effectively generates a longer CaM binding domain in CaM kinase II (Fig. 6, B and C).

A result from this study that could not have been anticipated by peptide studies is that the F293A and N294A mutations of CaM kinase II slow dissociation of CaM, by 15- and 13-fold, respectively, from unphosphorylated kinase (Fig. 4B). This suggests that the wild-type side chains destabilize the CaM kinase II/CaM complex when kinase is unphosphorylated, which is the opposite of their stabilizing effect on the trapped state (Fig. 4B). A structural explanation for this result is illustrated in Fig. 6. When CaM kinase II is unphosphorylated, Phe293 and Asn294 make strong interactions with Phe86 and Ile205 in the catalytic core (37) (Fig. 6A). These interactions are weakened in the CaM-bound form (Fig. 6B). Thus, the roles of Phe293 and Asn294 in the absence of phosphorylation are to stabilize the autoinhibited form of the enzyme relative to the CaM-bound complex, shifting the equilibrium toward the dissociated components, whereas when Thr286 is phosphorylated, Phe293 and Asn294 stabilize the CaM-bound form of the enzyme (Fig. 6, B and C). Therefore, the Phe293 and Asn294 side chains can either inhibit or promote CaM dissociation, depending on whether the kinase is autophosphorylated.

Trapping may be described in terms of the effect of phosphorylation on the equilibrium between free kinase and calmodulin on one side, and the CaM kinase II/CaM complex on the other. A important question for trapping and for other processes that are regulated by phosphorylation is: how does phosphorylation change the equilibrium so dramatically? In CaM kinase II, Phe293 and Asn294 stabilize both sides of this equilibrium by making independent sets of interactions on either side. Phosphorylation disrupts the set of interactions between Phe293 and Asn294 and the catalytic core, and at the same time,
it enables these residues to interact with CaM. This mechanism approximately doubles the contribution of these side chains to the change in equilibrium after autophosphorylation and explains a significant share of the dramatic effect of phosphorylation. In Phe293 and Asn294 mutants, \( k_{\text{off}} \) only decreases 160- and 330-fold after autophosphorylation, as opposed to the 23,000-fold observed for wild-type. Similar mechanisms, whereby phosphorylation selectively perturbs residues that are energetically critical on both sides of a two-state system, may underlie the switch-like effects in other systems as well.

The relative contribution of specific residues to the interaction with the kinase or CaM can be examined by the use of \( \Delta \Delta G \). Although we have not determined equilibrium constants, we make the assumption that changes in on-rates are small in comparison to the off-rates that we determined, as is generally observed (29–32). Phosphorylation of CaM kinase II is characterized by a 23,000-fold effect on the dissociation rate constant of CaM (Table II), which is equivalent to a \( \Delta \Delta G \) value of 6.1 kcal mol\(^{-1} \). The \( k_{\text{off}} \) values are 0.002 kcal mol\(^{-1} \) and \( \Delta \) is 303 K. For the P293A, N294A, and R297E mutants, the effect of phosphorylation is reduced by 3.0, 2.6, and 1.1 kcal mol\(^{-1} \), respectively. Taken together, these residues may account for the effect of phosphorylation on the dissociation rate constant, although coupling may reduce their combined effect. On the other hand, we cannot account for the full effect of trapping based on the CaM residues that we have studied. We obtained a 116-fold effect (2.9 kcal mol\(^{-1} \)) from CaM (E120A/M124A)/ kinase(R297E) compared with the wild-type complex (Table II). A factor of 210 (3.2 kcal mol\(^{-1} \)) is missing on the CaM side of the binding interface, and we may have failed to identify one or a few CaM residues that are important for trapping, although we have identified several residues that are not involved. Alternatively, the structural plasticity of CaM (16) may allow conformational changes that compensate for the effects of mutations. Such a mechanism could cause the free energy of trapping to be distributed over a large number of CaM residues.

The present study demonstrates that Phe293 in CaM kinase II and Glu120 and Met124 in CaM are also critical for CaM trapping following a drop of \([\text{Ca}^{2+}]\) to low levels (Fig. 2). Thus, these residues likely also mediate the increased affinity of CaM for \( \text{Ca}^{2+} \) when CaM is bound to CaM kinase II. CaM trapping in low \([\text{Ca}^{2+}]\) may underlie the ability of CaM kinase II to detect the frequency of \( \text{Ca}^{2+} \) oscillations (10), and our study implicates Phe293 and Asn294 as important residues for \( \text{Ca}^{2+} \) spike frequency detection. Mutations at these sites result in slower dissociation of CaM from the unphosphorylated kinase and faster dissociation from the phosphorylated from. As a result, the modified forms of CaM kinase II are predicted to become trapped at a lower spike frequency and to yield a less persistently trapped form than wild-type CaM kinase II, thus causing a broadening of the frequency response function. We are currently testing these predictions by exposing complexes containing our CaM kinase II mutants to calcium oscillations in vitro, as has been done for wild-type CaM and CaM kinase II (10). The effects of such kinetically well characterized variants in transgenic mice could yield significant insights into the processes of long term potentiation, learning, and memory (42–45).
Molecular Characterization of Calmodulin Trapping by Calcium/Calmodulin-dependent Protein Kinase II
Sheela I. Singla, Andy Hudmon, Jonathan M. Goldberg, Janet L. Smith and Howard Schulman


Access the most updated version of this article at doi: 10.1074/jbc.M101744200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 25 of which can be accessed free at http://www.jbc.org/content/276/31/29353.full.html#ref-list-1