The IQ-motif protein PEP-19, binds to the C-domain of calmodulin (CaM) with significantly different $k_{on}$ and $k_{off}$ rates in the presence and absence of Ca$^{2+}$, which could play a role in defining the levels of free CaM during Ca$^{2+}$ transients. The initial goal of the current study was to determine whether Ca$^{2+}$ binding to sites III or IV in the C-domain of CaM was responsible for affecting the kinetics of binding PEP-19. EF-hand Ca$^{2+}$-binding sites were selectively inactivated by the common strategy of changing Asp to Ala at the X-coordination position. Although Ca$^{2+}$ binding to both sites III and IV appeared necessary for native-like interactions with PEP-19, the data also indicated that the mutations caused undesirable structural alterations as evidenced by significant changes in amide chemical shifts for apoCaM. Mutations in the C-domain also affected chemical shifts in the unmodified N-domain, and altered the Ca$^{2+}$ binding properties of the N-domain. Conversion of Asp$^{93}$ to Ala caused the greatest structural perturbations, possibly due to the loss of stabilizing hydrogen bonds between the side chain of Asp$^{93}$ and backbone amides in apo loop III. Thus, although these mutations inhibit binding of Ca$^{2+}$, the mutated CaM may not be able to support potentially important native-like activity of the apoprotein. This should be taken into account when designing CaM mutants for expression in cell culture.

PEP-19 is a small (62 amino acids) protein that binds to calmodulin (CaM) in the presence or absence of Ca$^{2+}$ (1) via an IQ CaM binding motif. Although PEP-19 has no known intrinsic activity other than binding to CaM, expression of PEP-19 can protect cells from apoptosis (2, 3), and cell death from Ca$^{2+}$ activity other than binding to CaM, expression of PEP-19 is important. The abbreviations used are: CaM, calmodulin; 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; DDPMA, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; HEDTA, N-(2-hydroxyethyl)ethylenedinitriotropic acid; NTX, nitrilo-t,2-2",3"-triacetic acid; HSQC, heteronuclear single quantum coherence; FRET, fluorescence resonance energy transfer; MOPS, 4-morpholinepropanesulfonic acid.

**EXPERIMENTAL PROCEDURES**

**Materials**—[15N]NH$_4$Cl was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Quin-2 (free acid), 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), and 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) were purchased from In Vitrogen. N-(4-Dimethyl-
amino-3,5-dinitrophenyl)maleimide (DDPM) was purchased from Alfa Aesar.

Expression and Purification of CaM Proteins—A pET23 expression vector with a mammalian CaM cDNA optimized for bacterial codon usage was kindly provided by Andy Hudmon at the Indiana School of Medicine. QuikChange® II XL Site-directed Mutagenesis Kit (Stratagene) was used to generate all mutant proteins. Calcium binding was inhibited by mutation of the X Ca$^{2+}$-coordinating site (see Fig. 1), which is the first of 12 amino acids in a canonical EF-hand Ca$^{2+}$-binding loop (32). In addition, a panel of Ca$^{2+}$-binding mutants with Lys$^{-}$ converted to Cys as described previously (33) was generated for labeling with fluorescent probes. A cDNA encoding human PEP-19 was synthesized with optimal bacterial codon usage (DNA2.0 Inc.) and subcloned into a pET23 expression vector.

Measurement of Calcium Binding Affinity of CaM Mutants—A FRET-based assay described previously (5) was used to measure calcium binding affinity of CaM to PEP-19. Typically, a solution of 1.0 μM 1,5-IAEDANS-labeled CaM(K75C) with 10.0 μM DDPM-labeled PEP-19 was prepared in a base buffer containing 20 mM MOPS, pH 7.5, 100 mM KCl, with either 2 mM CaCl$_2$ or 1 mM EGTA. A concentrated stock solution of unlabeled native CaM (control) or CaM mutant in the same solution was used to titrate the complex. Apparent IC$_{50}$ values were derived by plotting the fluorescence response versus log(competitor) and fitting to the following equation.

\[
F = F_0 + A_1 \left( \frac{C_{a_{i}}^{n}}{C_{p_{i}}^{n} + K_1'} \right) + A_2 \left( \frac{C_{a_{j}}^{n}}{C_{p_{j}}^{n} + K_2'} \right) \quad \text{(Eq. 1)}
\]

Where $F_0$ is the fluorescence without Ca$^{2+}$, $C_{a_{i}}$ is the free Ca$^{2+}$ level, $A_i$ is the amplitude of change due to a given binding event, $n$ is the Hill coefficient, and $K$ is the $K_{Ca^{2+}}$.

Stopped-Flow Measurements—Experiments were performed at 23 °C using an Applied Photophysics Ltd. (Leatherhead, UK) model SX20 15 MV sequential stopped-flow spectrophotofluorometer with a 150 watt Xe/Hg lamp. The system was configured with two 3-ml syringes (A and B) and has a dead time of 1.7 ms, all solutions contained a base buffer of 20 mM MOPS, pH 7.5, 100 mM KCl. Three independent fluorescence indicators were used to measure the rate of dissociation ($k_{off}$) of Ca$^{2+}$ from CaM or CaM mutants; intrinsic tyrosine, 1,8-ANS, and Quin-2. For Tyr fluorescence, syringe A contained 4 μM CaM and 0.1 mM CaCl$_2$, whereas syringe B contained either 10 mM EGTA or 0.1 mM CaCl$_2$ as a negative control for each CaM. The optical/mixing chamber was excited at 276 nm and fluorescence was collected using Oriel filter 51662. Changes in fluorescence obtained with Ca$^{2+}$ in syringe B were subtracted from data obtained with EGTA in the syringe. Experiments using 1,8-ANS were performed as for Tyr, but with 4 μM 1,8-ANS included in syringe A. 1,8-ANS was excited at 366 nm and fluorescence was collected using a 430-nm cutoff filter (Oriel 51282). Measurements using Quin-2 included 4 μM CaM and 30–60 μM CaCl$_2$ in syringe A, and 200–400 μM Quin-2 in syringe B. The samples were excited at 334.5 nm and fluorescence was collected using a 430-nm cutoff filter (Oriel 51282). Data collected using all three fluorescent markers fit well to an equation with a single exponential rate constant. Fluorescence from Quin-2 was calibrated by replacing CaM in syringe A with 2, 4, 6, 8, or 10 μM EGTA. The amplitude of the slow rate of release of Ca$^{2+}$ from EGTA ($k_{off} = 0.7$ s$^{-1}$) could be readily used to calibrate the fluorescence response.

NMR Methodology—All NMR experiments were collected on a DRX 600 MHz spectrometer instrument using a 5-mm TXI Cryprobe at 298 K for apo samples and 310 K for Ca$^{2+}$ samples. The data were processed using FELIX 2007 software. Resonance assignments were made by comparison to wild-type CaM. For experiments requiring decalcified proteins, proteins were decalcified by adding 1 to 5 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid followed by desalting on a Bio-Gel P-10 column equilibrated in buffers that were decalcified by passage over a Calcium Sponge column (Molecular Probes). The decalcified proteins were lyophilized and resuspended in decalcified buffer containing 10 mM imidazole, 100 mM KCl, 5% D$_2$O at pH 6.3. Calcium was added to 20 mM to the $^{15}$N-apoCaM mutants and the pH adjusted accordingly. Chemical shift perturbation was calculated using the following equation.

\[
F = F_0 + A_1 \left( \frac{C_{a_{i}}^{n}}{C_{p_{i}}^{n} + K_1'} \right) + A_2 \left( \frac{C_{a_{j}}^{n}}{C_{p_{j}}^{n} + K_2'} \right) \quad \text{(Eq. 2)}
\]

Where $F_0$ is the fluorescence without Ca$^{2+}$, $C_{a_{i}}$ is the free Ca$^{2+}$ level, $A_i$ is the amplitude of change due to a given binding event, $n$ is the Hill coefficient, and $K$ is the $K_{Ca^{2+}}$. 

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Where $\Delta \delta H$ is the change in $^1$H chemical shift and $\Delta \delta N$ is the change in $^{15}$N chemical shift.

RESULTS

Mutation at $Ca^{2+}$-binding Sites Alters Binding of PEP-19 to CaM—Fig. 1 shows the consensus sequence of a canonical EF-hand $Ca^{2+}$-binding loop (32), which consists of 12 amino acids that provide 7 coordination positions for $Ca^{2+}$. All mutated CaMs used in this study have this Asp converted to Ala at position 1 as indicated by the arrowhead. Nomenclature for mutant CaM proteins was adopted from the literature (34). For example, CaM3,4 indicates that Asp93 and Asp129 were changed to Ala at the X coordination position of $Ca^{2+}$-binding loops III and IV, respectively.

We first determined the relative affinity of PEP-19 for native CaM and the CaM mutant proteins, with a focus on CaM3, CaM4, and CaM3,4 because PEP-19 binds selectively to the C-domain of CaM. CaM with Lys75 changed to Cys was labeled with the FRET donor 1,5-IAEDANS (CaMδ), and PEP-19 with a C-terminal Gly-Cys extension was labeled with the FRET acceptor DDPM (PEP-19A). The relative affinity of the CaM mutants for PEP-19 in the presence or absence of $Ca^{2+}$ was determined by their ability to compete with CaMδ for binding to PEP-19A. Because PEP-19 binds to CaM with similar affinity in the presence or absence of $Ca^{2+}$ (6), we anticipated that inactivation of $Ca^{2+}$-binding sites would have little effect on binding affinity. However, Fig. 2 shows that only $Ca^{2+}$-CaM3 had similar affinity for PEP-19 as native $Ca^{2+}$-CaM (see Fig. 2A), all other mutants had lower affinity in either the presence or absence of $Ca^{2+}$ as indicated by a rightward shift in the binding curves. Results shown in Fig. 2B in the absence of $Ca^{2+}$ are particularly interesting. If the mutations inhibit or prevent $Ca^{2+}$ binding to a given EF-hand in CaM, but do not greatly
alter the structure of CaM in the absence of Ca\(^{2+}\), then all mutant proteins should have similar affinity for PEP-19 in the apo forms. In contrast, the apo forms of all mutant proteins have lower affinity for PEP-19 relative to native apoCaM.

We showed previously that the \(k_{\text{on}}\) and \(k_{\text{off}}\) rates for binding PEP-19 to Ca\(^{2+}\)-CaM are too rapid to measure using a stopped-flow fluorimeter (6). Fig. 3A shows that mutation of sites III, IV, or both III and IV greatly decrease the rate of dissociation of PEP-19 from CaM in the presence of Ca\(^{2+}\). This indicates that Ca\(^{2+}\) binding to both sites III and IV are necessary to allow a fast rate of dissociation of PEP-19 from CaM.

In contrast to Ca\(^{2+}\)-CaM, both \(k_{\text{on}}\) and \(k_{\text{off}}\) rates for binding PEP-19 to apoCaM are slow and can be easily measured. Rates for mutant proteins should be identical to CaM if mutation of the Ca\(^{2+}\)-binding loops does not alter the structure of CaM, however, Fig. 3B shows this is true only for CaM4. The \(k_{\text{off}}\) for CaM3 and CaM3,4 are 3- to 4-fold greater than native CaM. Based on the data in Figs. 2B and 3B, the \(k_{\text{on}}\) rates (\(\mu\text{M}^{-1}\,\text{s}^{-1}\)) for binding PEP-19 are: CaM = 0.36; CaM3 = 0.45; CaM4 = 0.15; CaM3,4 = 0.59. Thus, differences in affinity of binding PEP-19 to the mutant proteins can result from changes in \(k_{\text{on}}\) or \(k_{\text{off}}\).

**Mutation of CaM-binding Sites Causes Structural Changes**

**FIGURE 3.** Rate of dissociation of PEP-19 from native CaM versus Ca\(^{2+}\)-binding mutants. Dissociation of CaM from PEP-19 was determined using a stopped-flow fluorimeter as described under “Experimental Procedures.” Essentially, donor-labeled CaM (1 \(\mu\text{M}\)) bound to acceptor-labeled PEP-19 (10 \(\mu\text{M}\)) was rapidly diluted to achieve partial dissociation of the complex, and an increase in fluorescence. Syringe A contained labeled CaM (1 \(\mu\text{M}\)) and labeled PEP-19 (10 \(\mu\text{M}\)) with 1 mM EGTA or 2 mM CaCl\(_2\). Syringe B contained 1 mM EGTA or 2 mM CaCl\(_2\), but no proteins. Panel A shows the dissociation in the presence of Ca\(^{2+}\) (2 mM CaCl\(_2\)). Dissociation from native Ca\(^{2+}\)-CaM is too fast to detect using a stopped flow with a 1.7-ms dead time. Panel B shows dissociation in the absence of Ca\(^{2+}\) (1 mM EGTA).

**FIGURE 4.** Calcium-binding mutations alter intrinsic Tyr fluorescence and 1,8-ANS binding. Panel A shows Tyr fluorescence in the presence and absence of Ca\(^{2+}\). Solutions contained 4 \(\mu\text{M}\) CaM, 50 mM MOPS, pH 7.5, 100 mM KCl, 1 mM EGTA or 1 mM CaCl\(_2\). Panel B shows fluorescence from 1,8-ANS, which fluoresces upon binding to hydrophobic surfaces. Conditions were the same as in panel A but with 4 \(\mu\text{M}\) 1,8-ANS.

**FIGURE 4.** Calcium-binding mutations alter intrinsic Tyr fluorescence and 1,8-ANS binding. Panel A shows Tyr fluorescence in the presence and absence of Ca\(^{2+}\). Solutions contained 4 \(\mu\text{M}\) CaM, 50 mM MOPS, pH 7.5, 100 mM KCl, 1 mM EGTA or 1 mM CaCl\(_2\). Panel B shows fluorescence from 1,8-ANS, which fluoresces upon binding to hydrophobic surfaces. Conditions were the same as in panel A but with 4 \(\mu\text{M}\) 1,8-ANS.

We showed previously that the \(k_{\text{on}}\) and \(k_{\text{off}}\) rates for binding PEP-19 to Ca\(^{2+}\)-CaM are too rapid to measure using a stopped-flow fluorimeter (6). Fig. 3A shows that mutation of sites III, IV, or both III and IV greatly decrease the rate of dissociation of PEP-19 from CaM in the presence of Ca\(^{2+}\). This indicates that Ca\(^{2+}\) binding to both sites III and IV are necessary to allow a fast rate of dissociation of PEP-19 from CaM.

In contrast to Ca\(^{2+}\)-CaM, both \(k_{\text{on}}\) and \(k_{\text{off}}\) rates for binding PEP-19 to apoCaM are slow and can be easily measured. Rates for mutant proteins should be identical to CaM if mutation of the Ca\(^{2+}\)-binding loops does not alter the structure of CaM, however, Fig. 3B shows this is true only for CaM4. The \(k_{\text{off}}\) for CaM3 and CaM3,4 are 3- to 4-fold greater than native CaM. Based on the data in Figs. 2B and 3B, the \(k_{\text{on}}\) rates (\(\mu\text{M}^{-1}\,\text{s}^{-1}\)) for binding PEP-19 are: CaM = 0.36; CaM3 = 0.45; CaM4 = 0.15; CaM3,4 = 0.59. Thus, differences in affinity of binding PEP-19 to the mutant proteins can result from changes in \(k_{\text{on}}\) or \(k_{\text{off}}\).

**Mutations in the C-domain Affect Ca\(^{2+}\) Binding to the N-domain**—The data in Figs. 2 and 3 suggest that conversion of Asp\(^{93}\) and/or Asp\(^{129}\) to Ala causes undesirable structural perturbations in CaM. Thus, we performed a series of experiments to characterize the mutant proteins, especially CaM3,4. We first compared the Ca\(^{2+}\) dependence of fluorescence from Tyr\(^{99}\) and Tyr\(^{138}\) in the C-domain of CaM. Fig. 4A shows that CaM and CaM1,2 respond similarly to Ca\(^{2+}\), indicating that...
mutations in the N-domain do not greatly affect Ca\(^{2+}\)-dependent Tyr fluorescence from the C-domain. Interestingly, Tyr fluorescence from both CaM3,4 and CaM3 showed little response to Ca\(^{2+}\), but had increased basal Tyr fluorescence in the absence of Ca\(^{2+}\). This suggests a more hydrophobic environment for Tyr in apo forms of CaM3 and CaM3,4 relative to native apoCaM.

We next compared exposure of hydrophobic surfaces in CaM and mutant proteins using 1,8-ANS, which fluoresces upon binding to hydrophobic surfaces. Fig. 4B shows much greater fluorescence from 1,8-ANS in the presence of Ca\(^{2+}\)-CaM versus apoCaM. In contrast, there is little change in 1,8-ANS fluorescence when Ca\(^{2+}\) is added to CaM1,2, indicating that the increase in 1,8-ANS fluorescence in the presence of native CaM is primarily due to Ca\(^{2+}\) binding to sites I and II in the N-domain. This is confirmed by CaM3,4, which does not bind Ca\(^{2+}\) to sites III and IV based on Tyr fluorescence (see Fig. 4A), but has a robust Ca\(^{2+}\)-dependent increase in ANS fluorescence upon binding Ca\(^{2+}\). The increase is much greater than observed with native CaM, suggesting that mutation of sites III and IV in the C-domain alters exposure of hydrophobic surfaces in response to Ca\(^{2+}\) binding to sites I and II in the N-domain. This is also observed for CaM3, but to a much lesser extent with CaM4.

Relative Ca\(^{2+}\)-binding affinities were determined by monitoring Tyr or 1,8-ANS fluorescence during titration of CaM and mutant derivatives with Ca\(^{2+}\). The results shown in Fig. 5 and Table 1 can be interpreted with respect to binding Ca\(^{2+}\) to the C- and N-lobes with \(K\text{-Ca}\) values of around 2 and 13 \(\mu\text{M}\), respectively (5, 35, 36). Tyr fluorescence detects a single class of Ca\(^{2+}\) binding sites in native CaM with \(K\text{-Ca}\) of 2.5 \(\mu\text{M}\), which corresponds with Ca\(^{2+}\) binding to the C-domain. Fluorescence data for native CaM collected using 1,8-ANS fit well to a single class of binding sites, with a \(K\text{-Ca}\) of 5.2 \(\mu\text{M}\). We interpret this \(K\text{-Ca}\) to reflect binding to sites I and II because Fig. 4 shows that 1,8-ANS is most sensitive to Ca\(^{2+}\) binding to the N-domain. The lower \(K\text{-Ca}\) relative to the native CaM may be due to a contribution to fluorescence from the C-domain, or an increase in Ca\(^{2+}\) binding affinity of the N-domain due to 1,8-ANS binding.

Both Tyr and 1,8-ANS fluorescence detect a single class of Ca\(^{2+}\)-binding sites in CaM1,2 with a \(K\text{-Ca}\) of about 2 \(\mu\text{M}\), which is consistent with Ca\(^{2+}\) binding to the C-domain. Although CaM3,4 shows no Ca\(^{2+}\)-dependent change in Tyr fluorescence, 1,8-ANS detects a single binding component with a \(K\text{-Ca}\) of 2.1 \(\mu\text{M}\). This is due to Ca\(^{2+}\) binding to the N-domain because NMR data show that Ca\(^{2+}\) does not bind to the C-domain of CaM3,4. The \(K\text{-Ca}\) of 2.1 \(\mu\text{M}\) is less than for the N-domain of native CaM by about 11 \(\mu\text{M}\) (5, 35, 36). Thus, mutation of Ca\(^{2+}\) binding sites III and IV increases the affinity of Ca\(^{2+}\)-binding to sites I and II in the N-domain.

Fig. 5B and Table 1 show that CaM3 and CaM4 have biphasic Ca\(^{2+}\) binding curves. Although values derived using Tyr and 1,8-ANS fluorescence are not identical, the trends are clear. Barraging some unusual cooperative interactions between N- and C-domain EF-hands, the simplest interpretation of the data is that the higher affinity site(s) associated with a large increase in 1,8-ANS fluorescence is due to binding Ca\(^{2+}\) at sites I and II in the N-domain of CaM3 and CaM4 because the Hill coefficient is close to 2. The lower affinity class of binding sites has a \(K\text{-Ca}\) close to 2. The lower affinity class of binding sites has a \(K\text{-Ca}\) close to 2.
Mutation of CaM-binding Sites Causes Structural Changes

Dissociation of CaM, which responds to Ca$^{2+}$, is too fast to be detected by stopped-flow methods. This fast phase is not seen for CaM1,2, because both N-domain sites are inactivated. Both CaM and CaM1,2 show a slower dissociation of 2 Ca$^{2+}$/mol of protein with similar rates of 10–12 s$^{-1}$, which is consistent with release of Ca$^{2+}$ from sites III and IV in the C-domain. These data, together with Table 1, show that mutation of sites I and II do not affect Ca$^{2+}$ binding to sites III and IV.

Very similar data were obtained using CaM3,4 and CaM3. Neither protein showed a detectable change in Tyr fluorescence, indicating an insensitivity to Ca$^{2+}$ release, or that the change occurs too rapidly to measure using stopped-flow techniques. Data collected with Quin-2 and 1,8-ANS fit single exponential decays with rates of between 200 and 300 s$^{-1}$, and stoichiometry of 2 Ca$^{2+}$/mol of protein. The simplest explanation for these data is that Quin-2 and 1,8-ANS detect dissociation of Ca$^{2+}$ from the N-domain of CaM. This would mean that mutation of C-domain Ca$^{2+}$-binding sites decreases the rate of Ca$^{2+}$ dissociation from the N-domain, and would account for the increase in affinity of Ca$^{2+}$ binding to the N-domain in CaM3 and CaM3,4 shown in Table 1.

Dissociation of Ca$^{2+}$ from CaM4 shows a single rate of 40 to 60 s$^{-1}$. There are two possibilities for this result. One is that the observed rate is associated with Ca$^{2+}$ release from the active site III in CaM4, and that release of Ca$^{2+}$ from sites I and II in the N-lobe is too rapid to detect. The other possibility is that release of Ca$^{2+}$ from the active site III is too fast to be observed, and that the observed rate is associated with dissociation of Ca$^{2+}$ from the N-domain, which is sensed by Tyr residues due to mutation of site IV in CaM4. The latter seems more plausible because: 1) low affinity binding of Ca$^{2+}$ to site III would likely be associated with a rapid rate of dissociation; 2) rapid release of Ca$^{2+}$ from the N-lobe would be detected as a large decrease of 1,8-ANS fluorescence during the dead time of the stopped-flow fluorimeter, but this is not observed; and 3) the stoichiometry of

![FIGURE 6. Effect of mutations on the rates of Ca$^{2+}$ dissociation. Dissociation of Ca$^{2+}$ ($k_{off}$) from the various CaMs was measured using fluorescence from 1,8-ANS, Tyr, and Quin-2 as described under "Experimental Procedures." All fluorescent markers gave very similar $k_{off}$ values as shown in Table 2. The arrow in panel A indicates very rapid release of Ca$^{2+}$ from CaM, which occurs in the dead-time of the stop flow, indicating a $k_{off}$ of $>$500.](image-url)

| Method | $k_{off}$ (s$^{-1}$) | Ca$^{2+}$/protein |
|--------|------------------|-------------------|
| CaM    | Quin-2           | 9.5 ± 0.2         |
|        | 1,8-ANS         | 10.5 ± 0.1        |
|        | Tyr             | 10.3 ± 0.4        |
| CaM1,2 | Quin-2           | 9.7 ± 0.1         |
|        | 1,8-ANS         | 12.5 ± 0.2        |
|        | Tyr             | 9.8 ± 1.1         |
| CaM3,4 | Quin-2           | 296 ± 1           |
|        | 1,8-ANS         | 270 ± 9           |
|        | Tyr             | ND                |
| CaM3   | Quin-2           | 282 ± 11          |
|        | 1,8-ANS         | 217 ± 6           |
|        | Tyr             | ND                |
| CaM4   | Quin-2           | 59 ± 3            |
|        | 1,8-ANS         | 52 ± 2            |
|        | Tyr             | 40 ± 2            |

$^a$ ND indicates no detectable change in Tyr fluorescence.

The vertical arrow in Fig. 6A shows a rapid decrease of fluorescence from 1,8-ANS during the dead time of the stopped-flow fluorimeter when Ca$^{2+}$-CaM is rapidly mixed with EGTA. This is consistent with rapid dissociation of Ca$^{2+}$ from the N-domain of CaM, which is too fast to be detected by stopped-flow methods. This fast phase is not seen for CaM1,2, because both N-domain sites are inactivated. Both CaM and CaM1,2 show a slower dissociation of 2 Ca$^{2+}$/mol of protein with similar rates of 10–12 s$^{-1}$, which is consistent with release of Ca$^{2+}$ from sites III and IV in the C-domain. These data, together with Table 1, show that mutation of sites I and II do not affect Ca$^{2+}$ binding to sites III and IV.

a

of 30–50 μM, and Hill coefficient of less than 2. This is consistent with Ca$^{2+}$ binding to the remaining active site in the C-domain of CaM3 and CaM4, which would have lower affinity due to the absence of cooperativity (6).

Regardless of the microscopic assignment of Ca$^{2+}$-binding sites, Fig. 5 and Table 1 demonstrate interdomain effects of mutations. First, fluorescence from Tyr$^{99}$ and Tyr$^{138}$ in the C-domain, which is insensitive to Ca$^{2+}$ binding to the N-domain of native CaM, responds to Ca$^{2+}$ binding to the N-domain of native CaM, responds to Ca$^{2+}$ binding to the N-domain of CaM3 and CaM4 because biphasic Ca$^{2+}$ binding curves are observed. Second, the apparent affinity of Ca$^{2+}$ binding to sites I and II is increased for CaM3 and CaM3,4 relative to native CaM.

Effect of Ca$^{2+}$-binding Mutations on Rates of Ca$^{2+}$ Dissociation—We next determined the effect of mutations on the rates of Ca$^{2+}$ dissociation ($k_{off}$) using stopped-flow techniques to monitor fluorescence from 1,8-ANS, Tyr, and Quin-2. Data obtained with 1,8-ANS are shown in Fig. 6, and rates obtained with all methods are shown in Table 2.
Ca\(^{2+}\) release is consistent with release of 2 Ca\(^{2+}\) from the N-lobe rather than 1 Ca\(^{2+}\) from site III.

Together, the data in Figs. 5 and 6 and Tables 1 and 2 indicate that mutation of Ca\(^{2+}\)-binding sites III and IV in the C-domain can affect Ca\(^{2+}\) binding to sites I and II in the N-domain. This is most evident for CaM3,4 because it does not bind Ca\(^{2+}\) to the C-domain, and exhibits Ca\(^{2+}\) binding to the N-domain with higher affinity and slower \(k_{off}\) rate than native CaM.

NMR Spectra Indicate Altered Apo Structure in CaM3,4 and Interdomain Effects of Mutations—NMR was used to assess potential structural changes due to conversion of Asp\(^{93}\) and Asp\(^{129}\) to Ala. Fig. 7A compares a region of the \(^{1}H\)-\(^{15}N\) HSQC spectra for CaM (gray) and CaM3,4 (black) in the absence of Ca\(^{2+}\). The upper panel shows that amide resonances for residues in the N-domain of CaM3,4 (residues 1–75) are very similar to those of CaM, but amides in the C-domain (residues 76–148) are greatly affected by the mutations. The lower panel shows that major differences are also seen in the crowded central region of the spectra.

Mutation of Asp\(^{93}\) and Asp\(^{129}\) destabilizes the C-domain of CaM. Fig. 7 compares selected regions of \(^{1}H\)-\(^{15}N\) HSQC spectra collected for native CaM (gray) and CaM3,4 (black) in the absence of Ca\(^{2+}\). The upper panel shows that amide resonances for residues in the N-domain of CaM3,4 (residues 1–75) are very similar to those of CaM, but amides in the C-domain (residues 76–148) are greatly affected by the mutations. The lower panel shows that major differences are also seen in the crowded central region of the spectra.

The majority of N-lobe amide assignments for the mutant proteins could be made by comparison with native CaM, however, resonances in the N-domain were affected by mutations in the C-domain. Fig. 8 shows chemical shift differences between native apoCaM and the apo forms of CaM3, CaM4, and CaM3,4. Chemical shifts for amides in site II are most affected by mutations in sites III and IV, especially Asn\(^{60}\) and Gly\(^{61}\) in CaM3, CaM4, and CaM3,4, and Asp\(^{64}\) in CaM3,4 shown by the gray bars. These data are consistent with Figs. 4–6, which show that Ca\(^{2+}\) binding to the N-domain is altered as a consequence of mutations in the C-domain.

Fig. 6 suggests that mutation of sites III and IV in the C-domain of CaM affects the structure of the Ca\(^{2+}\)-bound N-domain because the rate of dissociation of Ca\(^{2+}\) from the N-domain of CaM3,4 is significantly slower than for native CaM. To further investigate this, the amide chemical shifts of CaM3,4 were followed during titration with Ca\(^{2+}\) and compared with native CaM. Fig. 9 shows chemical shift differences for residues that could be confidently assigned in the Ca\(^{2+}\)-bound N-domain of CaM3,4 versus native CaM. Observed differences are greater in magnitude than for the apoproteins shown in Fig. 8, and the greatest changes are clustered in the hydrophobic pocket as shown by the inset. A change in exposure of hydrophobic surfaces in the Ca\(^{2+}\)-bound N-domain due to mutations in the C-domain is consistent with the increased ANS fluorescence from CaM3,4 shown in Fig. 4B.

DISCUSSION

The original goal of this study was to determine whether Ca\(^{2+}\) binding to sites III or IV in the C-domain of CaM has dominant effects on the characteristics of binding PEP-19. The two most common strategies to inhibit Ca\(^{2+}\) binding to EF-hand motifs are: 1) convert Glu at loop position 12 to Gln, Lys, or Ala (7, 8) (see Fig. 1); and 2) convert Asp at loop position 1 to Ala (9). The latter mutation was used previously to inactivate Ca\(^{2+}\) binding to cardiac troponin C (10), skeletal troponin C (11), myosin regulatory light chain (12), and CaM (9). Mutation at loop position 1 in CaM has been used extensively to assess the relative functional contribution of Ca\(^{2+}\) binding to the N- and
C-domains of CaM when expressed in cell culture (13–31). Thus, we elected to convert Asp to Ala in the first ligand position in all Ca\(^{2+}\)/H\(^{11001}\)-binding mutants. Although the data in Figs. 2 and 3 indicate that Ca\(^{2+}\) binding to both sites III and IV of CaM is important for native-like interactions with PEP-19, apparent structural perturbations induced by the mutations complicated interpretation of the data. This led us to characterize the effect of these mutations on the Ca\(^{2+}\)/H\(^{11001}\) binding properties and structure of CaM.

Data in Figs. 5 and 6 and Tables 1 and 2 show that mutation of Ca\(^{2+}\)-binding sites in the C-domain increases the Ca\(^{2+}\)/H\(^{11001}\)-binding affinity of the N-domain by decreasing the rate of Ca\(^{2+}\) dissociation from the N-domain (37, 38), and that mutation of Val\(^{136}\) to Gly in site IV increases the Ca\(^{2+}\) binding affinity of the N-domain (39).

NMR analyses in Figs. 7–9 show that mutation of Ca\(^{2+}\)-binding sites in the C-domain causes significant changes in amide chemical shifts that are consistent with a loss of structure. In addition, mutations in the C-domain affect amide chemical shifts in the N-domain. There are at least two general mechanisms for this observation. One involves a specific interaction between domains that are disrupted by mutations. A recent crystal structure of Ca\(^{2+}\)-CaM shows a collapsed structure in which helix A in the N-domain makes significant H-bonding interactions with helices G and H in the C-domain (40). Such a conformation would undoubtedly be a minor species in solution. Moreover, the apparent \(K_{Ca^{2+}}\) for Ca\(^{2+}\) binding to isolated N and C domains of CaM are very similar (<1.5-fold different) to those determined for the intact protein (35, 36, 41, 42). This would not be expected if the Ca\(^{2+}\) binding properties of the N- and C-domains of intact CaM experience an intrinsic effect of domain-domain interactions.

A second mechanism for interdomain effects of mutations involves relatively nonspecific interactions between the N-domain and the mutated C-domain. Even though a highly flexible tether links these domains, it is reasonable that random arcing motions would allow nonspecific collisions. Changes in the surface chemistry due to mutations could lead to enhanced interactions between domains. Fig. 9 shows that residues in the hydrophobic pocket of the Ca\(^{2+}\)-bound N-domain are most affected by mutation of Asp\(^{93}\) and Asp\(^{129}\) in the C-domain. This supports a model in which exposed surfaces in the mutated C-domain associate with the N-terminal hydrophobic pocket in the presence of Ca\(^{2+}\) to stabilize the Ca\(^{2+}\)-bound form, and increase Ca\(^{2+}\) binding affinity by decreasing the \(k_{off}\).

Interestingly, inactivation of sites I and II in CaM1,2 does not affect Ca\(^{2+}\) binding to the C-domain. Because the overall affin-
Mutation of CaM-binding Sites Causes Structural Changes

FIGURE 10. NMR solution structures for apoCa2+-binding loops III and VI. Solution structure coordinates were taken from Kuboniwa et al. (45). Asp93 and Asp129 are the first amino acids in loops III and IV, respectively, and provide the X Ca2+ coordination ligand. Glu104 and Glu140 are the 12 amino acids in the loops and provide the −Z coordination position. Yellow lines indicate backbone amide hydrogens that are within 2 to 3 Å of carboxyl side chain of Asp93.

The extent of structural perturbation in the apo state as summarized above. Specifically, the apo C-domain is less stable than the N-domain and may be more sensitive to mutations.

This raises the question of what is the best way to mutate Ca2+-binding sites of EF-hand proteins for in vivo and in vitro studies? Mutation strategies must inhibit Ca2+ binding, but should also maintain structural integrity of the apo form to allow productive interactions with target proteins that require the apo state. The data presented here argue that mutation of the first ligand is not the best choice for inactivation of EF-hands III and IV in the C-domain of CaM. However, similar mutations may be suitable for the N-domain, which has greater intrinsic stability in the apo form.

Mutation of Glu at loop position 12 (see Figs. 1 and 10) has been used to inhibit Ca2+ binding to CaM (7, 8), because its side chain carboxyl group provides bidentate coordination of Ca2+ (32) (see Fig. 1) and it is solvent exposed in the apo form (44, 45). Mutation of Glu104 or Glu140 to Gln in sites III and IV, respectively, does not abolish Ca2+ binding, but greatly reduces affinity (50, 51). This is consistent with an analogous mutation in site I of skeletal troponin C, which allows weak Ca2+ binding via the first 5 coordination positions, but the EF-hand does not undergo a conformational change (52). Relative to CaM3 or CaM4, published NMR spectra of CaM with E104Q or E140Q mutations show fewer and less dramatic changes in amide chemical shifts in the C-domain (50, 51).

The potential to recover Ca2+ binding at mutated EF-hands is an important consideration, and this potential will likely depend on the nature of the mutation, the EF-hand protein being studied and its putative target proteins. For example, conversion of Asp to Ala at the Y coordination position in sites II, III, or IV of cardiac troponin C inhibited Ca2+ binding to the free protein, however, Ca2+ binding activity was recovered at sites III and IV, but not site II, when the mutant proteins were incorporated into a troponin complex (54).

In summary, mutation strategies for inhibition of Ca2+ binding to EF-hands must balance retention of structural integrity against the potential for recovery of Ca2+ binding activity upon interaction with native protein binding partners. This is especially problematic if the mutated proteins are to be expressed in cell culture where there is the potential for interaction with multiple binding proteins.

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