Kinetic Control of the Dissociation Pathway of Calmodulin-Peptide Complexes*

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The mechanism of dissociation reactions induced by calcium chelators has been studied for complexes of Drosophila calmodulin with target peptides, including four derived from the skeletal muscle myosin light chain kinase target sequence. Reactions were monitored by fluorescence stopped-flow techniques using a variety of intrinsic probes and the indicator Quin2. For most of the complexes, apparently biphasic kinetics were observed in several fluorescence parameters. The absence of any obvious relationship between dissociation rates and peptide affinities implies kinetic control of the dissociation pathway. A general mechanism for calcium and peptide dissociation was formulated and used in numerical simulation of the experimental data.

Unexpectedly, the rate of the slowest step decreases with increasing [peptide]/[calmodulin] ratio. Numerical simulation shows this step could contain a substantial contribution from a reversible relaxation process (involving the species Ca4-CaM-peptide), convolved with the following step (loss of C-terminal calcium ions). The results indicate the potentially key kinetic role of the partially calcium-saturated intermediate species. They show that subtle changes in the peptide sequence can have significant effects on both the dissociation rates and also the dissociation pathway. Both effects could contribute to the variety of regulatory behavior shown by calmodulin with different target enzymes.

Calmodulin is involved in the regulation of a range of cellular functions, usually through its Ca2+-dependent activation of target proteins (1). Ca4-CaM3 binds to many target proteins with high affinity (Kd ∼ nM) and binds peptides derived from the calmodulin binding regions of these proteins with similar affinities.

The x-ray crystal structure of Ca4-CaM (2–4) shows two globular domains with similar conformation, each containing two helix-loop-helix Ca2+ binding sites. Those in the C-domain have a higher affinity than those in the N-domain, and there is positive cooperativity between two sites within a domain (5). The crystal structure shows the two domains separated by an extended α-helix. In solution, this central helix contains a loop (residues 74–82) which allows the calmodulin domains to interact closely with the peptide (6).

Calcium binding to calmodulin induces a conformational change that exposes hydrophobic surfaces which comprise the binding site for target molecules. The solution structure of the complex of Ca4-CaM with M13, a 26-residue peptide derived from sk-MLCK, has been determined by NMR (7). The M13 peptide is in an α-helical conformation, effectively enclosed by the N- and C-domains of the calmodulin. The N and C termini of the peptide interact primarily with the C- and N-domains of calmodulin, respectively, and the Trp-4 and Phe-17 residues of the peptide appear to play an important anchoring role. The structures of the complexes of Ca4-CaM with peptides derived from sm-MLCK and CaM kinase II have been determined by x-ray diffraction (8, 9). The structures of the three complexes are rather similar, although there are significant differences in the positions of the peptides and the relative orientations of the calmodulin domains. In particular, the complex of Ca4-CaM with the CaM kinase II peptide has fewer peptide residues involved in helix formation and contact with the calmodulin, and the longer loop in the central helix region (residues 73–83) allows the domains to move closer together (9). The different structures show how calmodulin can adapt to bind peptides of different sequences, while maintaining high affinity in the interaction.

In recent work we studied the peptide WFF, which corresponds to residues 1–18 of M13 and contains the major sites of interaction with CaM (7). We have also permuted the sequence of WFF to include either Trp or Phe residues at positions 4, 8, and 17 (peptides WFW, FFW, and FFF; Table I). These peptides bind with high affinity to Ca4-CaM and retain the standard orientation with residues 4 and 17 interacting with CaM C- and N-domains, respectively (10, 11). They are therefore well suited for investigating the effect of controlled structural modifications on the kinetics and equilibria of CaM-target peptide interactions (12, 13).

In the present work we have measured the dissociation kinetics of seven Ca4-CaM-peptide complexes (14). The peptides studied are the four related to M13, the wasp venom peptides mastoparan and mastoparan X, and a synthetic peptide designed to form a basic amphiphilic α-helix (15). The effects of Ca2+ removal with a chelator were monitored using different fluorescent signals, including those of the chelator Quin2, peptide Trp, CaM Tyr (Tyr-138 in Drosophila CaM), and a Trp-containing calmodulin mutant, T26W (16, 17). Correlation of the kinetics observed with these different probes allows the deduction of a general mechanism for the dissociation of Ca4-CaM-peptide complexes. The results obtained are compared with other studies on the dissociation of CaM-peptide and CaM-protein complexes (18–24). We show that the predomi-
nent kinetic pathway is sensitive to changes in individual residues of the target peptides and deduce that the rate of the slowest step is determined by the contribution of a hitherto unsuspected kinetic relaxation mechanism involving the intermediate species Ca₃CaM-peptide, with two Ca²⁺ ions bound in the C-domain.

**Materials and Methods**

**Proteins and Peptides—**Drosophila melanogaster CaM was prepared as described (10). The T26W SYNcam mutant was a gift from Dr. J. Haecht. The peptides WFF, FFW, FFW, and CBP1 (Table I) were synthesized on an Applied Biosystems 430A peptide synthesizer and purified by reverse phase high performance liquid chromatography. The mastoparan was from Bachem. Peptide purity was assessed by mass spectroscopy and high performance liquid chromatography. All solutions were prepared in 25 mM Tris, 100 mM KCl (pH 8). Concentrations were determined using reported extinction coefficients for CaM (25) and calculated extinction coefficients at 259 or 280 nm for T26W and the peptides (26). The affinities of the peptides for CaM were measured by direct fluorometric titration (10) or by competition with a peptide of known affinity.

**Stopped-flow Measurements—**Stopped-flow experiments were performed on a Hi-Tech SF61-MX stopped-flow spectrophotometer. Trp, Tyr, and Quin2 fluorescence signals were monitored using excitation wavelengths of 290, 280, and 334 nm, respectively, and emission cut-on filters of 320, 305, and 370 nm, respectively. Concentrations quoted are those prior to 1:1 mixing. The instrument dead-time is ~3 ms at a drive pressure of about 4 bar (0.4 MPa). EGTA-induced dissociation was studied by mixing 0.5–2 μM CaM-peptide complex (in 100 mM Ca²⁺) with 20 mM EGTA. The observed rates were independent of [EGTA] in the range 1–20 mM. Quin2-induced dissociation was studied by mixing 3 μM CaM-peptide complex (in 20 mM Ca²⁺) with 90 μM Quin2. The observed rates were slightly dependent on [Quin2]. Dissociation induced by the addition of a silent peptide (CBP1 or FFF) was studied by mixing 0.5–1 μM CaM-peptide complex (in 100 μM Ca²⁺) with excess silent peptide. Finally, association reactions between CaM and peptide were studied by monitoring Trp fluorescence after mixing of 0.4 μM peptide with 0.4 μM Ca₃CaM (in 100 mM Ca²⁺).

**Data Analysis and Simulation—**For each reaction studied at least six stopped-flow traces were averaged for non-linear least squares analysis. A single exponential was considered satisfactory unless a better fit was obtained for a two-exponential fit. Rate constants were determined independently at least twice and are reported as mean ± S.D. The EGTA-induced dissociation of FFF monitored by Tyr fluorescence showed a pronounced lag phase and was fitted to the equation for the appearance of C in a first-order series reaction, A → B → C. Reactions showing only a small lag phase were fitted to a single exponential. Association reactions were studied under second-order conditions, and approximate values for dissociation rate constants (k_diss) were obtained by comparing the experimental curves with simulations for k_diss values in the range 0.1–5 × 10⁹ M⁻¹s⁻¹.

Numerical simulations were performed using the program KSIM (Runge-Kutta algorithm) and analyzed using the program KFPT (from Dr. N. Millar). Concentrations of complexes present prior to mixing were established by a short presimulation starting at the appropriate total concentrations of peptide and Ca₃CaM. The following assumptions were made for the simulations. All steps involving Ca²⁺ dissociation were taken to be irreversible because of the large excess of calcium ions in these studies. We therefore studied the association of WFF (0.4 μM) with Ca₄ CaM (0.4 μM) at 9.5 °C (Fig. 1D). The k_on value obtained as described under “Materials and Methods” was 1 ± 0.5 × 10⁹ M⁻¹s⁻¹. This is similar to a value of 9 × 10⁹ M⁻¹s⁻¹ found for the reaction of a fluorescently labeled CaM with two peptides derived from sm-MLCK (22) and to a value of 2 × 10⁹ M⁻¹s⁻¹ deduced from NMR studies on a peptide derived from Borde-tella pertussis adenylyl cyclase (30). These on-rates are close to the expected diffusion limit of 2 × 10⁹ M⁻¹s⁻¹ (31, 32). The high k_on has important consequences for subsequent mechanistic arguments (see “The Kinetic Model”). Although slow processes attributed to conformational changes have been observed in studies of the association of fluorescently labeled CaM with peptides and proteins (22, 33), no slow processes were observed in the association reactions studied here. Values of k_diss for the interaction of CaM with proteins tend to be lower (20, 21), but a value of 5.3 × 10⁸ M⁻¹s⁻¹ was measured for caldesmon (20).

**Calcium Dissociation from Calmodulin**

Rates for the dissociation of the C-terminal Ca²⁺ ions from Ca₄CaM (Table II) agree well with previous values (34–36). The rates observed for T26W SYNcam are two to three times faster than those for wild-type Drosophila CaM (Table II) and are somewhat faster than the value of 14 s⁻¹ reported for SYNcam (37). We note, however, that the slow phase rate observed with T26W only corresponds to some 5% of the total amplitude; the major signal change reflects dissociation of the N-terminal Ca²⁺ ions. These small differences may be due to sequence differences between the proteins (38).

**Dissociation of Ca₄CaM-Peptide Complexes**

Fig. 1 shows typical traces for the dissociation of the CaM complexes of the peptides WFF, FFF, and MaxX induced by excess chelator. A–C of Fig. 1 are representative of the range of kinetic behavior observed. The complexes clearly show diverse properties, when compared with one another for a given optical parameter or when compared for different optical parameters for the same complex. The results in Table II show rates and relative amplitudes for the complexes studied. Full discussion of these results requires a consideration of possible mechanisms (see “The Kinetic Model”). However, some generalizations can usefully be made here.

The rates observed with the complexes and Quin2 (Table II) are frequently biphasic, with a fast rate of 5–100 s⁻¹ and a slow rate of 0.5–5 s⁻¹. The total amplitude corresponds to four Ca²⁺ ions. The rates are markedly slower than those observed with CaM alone, where fast and slow rates clearly correspond to

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2. S. R. Martin, unpublished work.

3. S. E. Brown, unpublished observations.

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**Table I**

| Peptide sequences | k_diss (M⁻¹s⁻¹) |
|-------------------|----------------|
| WFF               | 3.0 ± 0.5      |
| FFW               | 1.5 ± 0.2      |
| FWF               | 0.5 ± 0.1      |
| MasX              | 0.1 ± 0.01     |
| Mas               | 0.05 ± 0.005   |
| CBP1              | 0.01 ± 0.001   |

**Note:** The Ca₄CaM-peptide complex was prepared with N-acetyl and carboxamido-protected N and C termini, respectively. All other peptides were unprotected.

4. E. Brown, unpublished observations.
dissociation from N- and C-domains, respectively (27). The presence of peptide enhances the affinity of both N- and C-domain Ca$^{2+}$ sites (see "The Kinetic Model"), and this assignment of the faster rate from the N-domain is evidently retained in the presence of peptide. By contrast, for FFW and FFF the Quin2 signals were monophasic, with a small lag phase in the case of Ca$_4$-CaM-FF (Fig. 1B). In this case it appears that a distinction between the N- and C-domain properties cannot be made.

For those cases showing biphasic Quin2 signals, the EGTA-induced dissociation of the complexes with the Trp-containing peptides also shows biphasic character, with the greater amplitude in the slow phase. The existence of the fast phase in the Trp signal in the case of WFF and FWF is an important observation, with mechanistic significance (see "The Relaxation Step: Experimental Justification"). It raises questions of the fluorescence properties of intermediate species and, more fundamentally, of the effects of coupling individual kinetic steps with different optical parameters. The biphasic kinetics observed in the EGTA dissociation of CaM-MasX is clearly exceptional, since (Fig. 1C) there is an intermediate species of enhanced fluorescence and the two kinetic components are oppositely signed.

The EGTA-induced dissociation of complexes of non-Trp-containing peptides with CaM shows a single phase, usually in the slow range, consistent with the origins of this signal at Tyr-138 in the C-domain of CaM. The EGTA-induced dissociation of Ca$_4$-CaM-FFF shows a lag phase, evidencing the sequential nature of the process.

The T26W mutant was used with all peptides, since it has a significant fluorescent change when Ca$^{2+}$ dissociates from the N-domain. In general, biphasic and monophasic behavior follow the Quin2 signals for all complexes (except CaM-Mas and CaM-CBP1). The rates are generally similar although CaM-MasX again appears exceptional. The relative amplitudes of biphasic transients are however different, with the fast phase predominating, consistent with the involvement of the N-domain. The signal in the case of complexes of T26W with Trp-containing peptides is composite, with similar contributions from the loss of Ca$^{2+}$ from the N-domain and the change due to complete dissociation of the peptide. These two contributions overlap and cannot readily be resolved.

There is no obvious relationship between the observed rates and the affinities of the peptides for CaM-CaM. Thus, for example, EGTA-induced dissociation rates for complexes with Mas or CBP1 are similar, even though the affinity of CBP1 for Ca$_4$-CaM is 2 orders of magnitude higher than that for Mas. Clearly the reactions studied here involve the eventual dissociation of both Ca$^{2+}$ and peptide from the initial complex, whereas the peptide affinities reflect only the association/dissociation reaction of the peptide from the complex with Ca$_4$-CaM. Thus, the pathway for the chelator-induced dissociation of the peptide appears to be under kinetic, rather than thermodynamic, control. Taken together, the results indicate that the dissociation mechanism is a complex multi-step pathway, with observed rates resulting from the coupling of individual kinetic steps.

The Kinetic Model

The purpose of the kinetic model is to account for the reduction in the Ca$^{2+}$ dissociation rates from CaM in the presence of peptides and to identify steps in the pathway where differences in the peptides produce significant kinetic effects. The complete kinetic scheme for the dissociation of Ca$^{2+}$ and peptide from a Ca$_4$-CaM-peptide complex becomes unduly complex if the four Ca$^{2+}$ ions are considered to dissociate independently. It is reasonable, however, based on extensive experimental evidence for the dissociation kinetics of Ca$_4$-CaM, to consider the Ca$^{2+}$ ions as dissociating in pairs, one pair from each domain (34). This assumption results in the Ca$^{2+}$ dissociation scheme shown in Fig. 2A, where C represents CaM and the subscripts P, N, and C represent peptide, and the N- and C-terminal Ca$^{2+}$ pairs, respectively. Therefore, for example, C$_{NCP}$ represents the full Ca$_4$-CaM-peptide complex, and C$_{CP}$ represents the Ca$_4$-CaM peptide complex with 2 Ca$^{2+}$ in the C-domain.

This kinetic scheme can be simplified given knowledge of the properties of CaM itself. As shown in Fig. 2A, step 2 of the path involves peptide dissociation from C$_{NCP}$ to produce the species C$_{NCP}$ (i.e., Ca$_4$-CaM). Dissociation of this species is known to proceed almost exclusively via steps 3 and 6, and not via steps 9 and 8, as the C-terminal Ca$^{2+}$ ions dissociate about one hundred times slower from Ca$_4$-CaM (C$_{NCP}$) than the N-terminal Ca$^{2+}$ ions (34). Correspondingly, we assume that dissociation of the C-terminal Ca$^{2+}$ pair from C$_{NCP}$ is always slower.
TABLE II

Observed rate constants (s−1) for the Quin2 and EGTA-induced dissociation of Ca2+ from Ca2-CaM and Ca4-CaM-peptide complexes

Experiments were performed at 20 °C in 25 mM Tris, 100 mM KCl (pH 8.0). Where two rates are given they are for the fast and slow phases, respectively. Errors in rates are ± S.D. For biphasic systems, the values in brackets are the amplitudes of the phases given as a percentage of the total amplitude.

| System       | Kd          | Quin2 EGTA (Trp) | EGTA (Tyr) | EGTA (T26W Trp) |
|--------------|-------------|-----------------|------------|-----------------|
| CaM          | 700 ± 200°  | NA              | 7.3 ± 0.5  | NA              |
|              | 8.5 ± 0.5   | NA              |            | 29 ± 5 (85°)    |
| T26W         | ≥800        | NA              |            | ≥800 (195°)     |
| CaM-WFF      | 0.12 9      | 10 ± 1 (30)     |            | NA              |
| CaM-FWF      | 6.5°        | 1.4 ± 0.1 (70)  | 1.5 ± 0.2 (90) | NA              |
| CaM-FFW      | 1.6°        | 3.3 ± 0.4 (55)  | 2.8 ± 0.4 (60) | NA              |
| CaM-FFF      | 1.0°        | 0.1 ± 0.1°      |            | 1.4 ± 0.5°      |
| CaM-MasX     | 0.9°        | 65 ± 5 (70)     | 91 ± 5°    | NA              |
| CaM-Mas      | 0.2°        | 1.3 ± 0.2 (30)  | 0.65 ± 0.05 | NA              |
| CaM-CBP1     | 0.005°      | 14 ± 1 (50)     |            | 1.4 ± 0.2       |

* The relative amplitudes of the phases could not be determined for the Quin2 dissociations of CaM and T26W as most of the signal for the fast phase was lost in the deadtime of the instrument.

* Not applicable.

* S. R. Martin, W. A. Findlay, and P. M. Bowley, manuscript in preparation.

* At 30 °C, 25 mM Tris, 100 mM KCl, 1 mM CaCl2, pH 7.5 (10).

* At 25 °C in 45 mM MOPS, 200 mM KCl, 1 mM CaCl2, pH 7.3 (29).

* The observed dissociations for the peptide FFF have a lag phase (Fig. 1B). The EGTA dissociation rates were determined using the equation for appearance of C in the reaction, A → B → C. The Quin2 signal was fitted to a single exponential (see “Materials and Methods”).

* Relative amplitudes are not given for this system because the fast and slow phases had amplitudes with opposite sign.

than dissociation of the N-terminal pair and have therefore eliminated step 12 and the subsequent steps 10 and 11. This reduces the scheme to two competing pathways (Fig. 2B).

In path A the N-terminal Ca2+ ions dissociate first (step 1), and in path B the peptide dissociates first (step 2). Path B is simply steps 2, 3, and 6. For path A, once the species C–CP forms it is possible for either the peptide to dissociate to form C–C– (step 4), or the C-terminal Ca2+ ions to dissociate to form C– (step 5). Step 4 is a reversible step and is a relaxation process in the mechanism, as opposed to a uni-directional irreversible step such as Ca2+ dissociation in the presence of a chelator. However, it is coupled to unidirectional steps 1 and 6 and is in parallel with step 5. It is necessary to know the relative rate values of these processes to determine which path will predominate.

The Relaxation Step: Theoretical Justification—The values of k−4 and k−5 can be estimated, and dissociation via step 4 is found likely to predominate in certain cases. Constants k−4 and k−5, the N- and C-terminal Ca2+ dissociation rates, can be estimated for a typical Ca2-CaM-peptide complex as follows. The interaction of Ca2-CaM with a peptide may be characterized by Equation 1,

\[ K'/K = (K'/K')_0 \]

where \( K_d \) and \( K_a \) are the dissociation constants for the interaction of the peptide with apo-CaM and Ca2-CaM. \( K \) and \( K' \) (average Ca2+ affinities) are equal to \( (K_d K_a/K_d K_a)_0 \) and \( (K_d K_a/K_d K_a)_0 \), respectively, where \( K_d \) and \( K_a \) are the stoichiometric Ca2+ association constants measured in the absence and presence of peptide (39).

The value of \( K \) for Drosophila CaM is 1.45 × 10^4 M−1 (25), and the values of \( K_a \) are known for each peptide (Table II). Values of \( K_d \) are less well established. Values of 620 and 25 mM have been reported for the peptides C28W and C20W from the CaM binding domain of the plasma membrane Ca2+ pump (40), and values of 80 mM and 5.7 μM have been reported for bovine heart phosphodiesterase and troponin I (41). Since binding of the peptides WFF and FFW to apo-CaM cannot be detected by CD at peptide concentrations of 100 μM, it is reasonable to assume a \( K_d \) of more than 1 mM for the sk-MLCK peptides. For a typical peptide with a \( K_d \) of 1 mM, values of \( K_d \) in the range 1 to 100 mM would correspond to \( (K'/K')_0 \) values in the range 10^6 to 10^8 and therefore to \( K'/K \) values in the range 30 to 100. Values in this range have been determined for other peptides (40, 41).

Stoichiometric Ca2+ association constants for CaM in the presence of the peptides used here show that both N- and C-domain Ca2+ affinities are enhanced, and consistent with this, peptide WFF (like other peptides) shows much lower affinity for Ca4-CaM (76 nM) than for Ca2-CaM (<0.2 mM) (13). Thus Ca2+ binding in both domains contributes to the enhanced peptide affinity, and the peptide affinity for a partially saturated Ca2-CaM is likely to be of the order of 10 M for Ca4-CaM. The above calculation can be taken further, assuming that the difference between \( K \) and \( K' \) is reflected in the Ca2+ dissociation rates. If the effect of the peptide on the N- and C-domain Ca2+ affinity is approximately equal, both the N- and C-terminal dissociation rates are expected to be decreased by the above factor of 30 to 100. Since the dissociation rates in the absence of peptide are ~700 s−1 (N-terminal) and 8.5 s−1 (C-terminal), we predict \( k_{−4} \) values in the range 25–7 s−1 and \( k_{−5} \) values in the range 0.3–0.085 s−1. The rate of Ca2+ dissociation from C–CP (step 5) is therefore predicted to be much slower than 8.5 s−1 (Table II), the rate of Ca2+ dissociation from CaM in the absence of peptide (step 6).

Calculations also show that \( k_{−4} \), the peptide dissociation rate from C–CP, is likely to be at least 10 s−1, owing to the lower affinity of the peptide for CaM after loss of the N-terminal Ca2+ ions, see above. By analogy with the \( k_{on} \) for the association of WFF with Ca4-CaM, \( k_a \) is likely to be of the order of 10^6 M−1 s−1, and hence the re-association reaction of the peptide becomes significant. Step 4 is reversible, i.e. it is a relaxation process. The rate of this step will be greater than \( k_{−4} \) and, since \( k_{−5} > k_{−4} \), it will exceed \( k_{−5} \). Hence, dissociation via step 4 should be significant. Even if the effect of the peptide is
largely on the C-terminal Ca\textsuperscript{2+} sites, both $k_{-4}$ and $k_{-8}$ will be reduced, and the relaxation step 4 and the dissociation step 5 will remain in competition.

The Relaxation Step: Experimental Justification—The reversible nature of step 4 suggests that the observed rates should be affected by the [peptide]/[CaM] ratio. We therefore measured the dissociation reaction for Ca\textsubscript{4}-CaM-WFF as a function of the [WFF]/[CaM] ratio. At [WFF]/[CaM] = 1.05 the EGTA-induced dissociation of Ca\textsubscript{4}-CaM-WFF (monitored by peptide Trp fluorescence) is biphasic with a fast phase rate of 12 s\textsuperscript{-1} and a slow phase rate of 1.5 s\textsuperscript{-1}. The fast phase accounts for approximately 10% of the total amplitude. At [WFF]/[CaM] > 1.5 the fast phase is no longer observed and the slow phase rate is reduced (see Fig. 3A and B).

Similarly, for Ca\textsubscript{4}-CaM-FWF, the fast phase (in Trp fluorescence) accounts for 40% at [peptide]/[CaM] = 1.05 (Table II) and decreases to 22% at [peptide]/[CaM] = 4.4 (Fig. 3B). Over this range the fast phase rate is unchanged (32 ± 4 s\textsuperscript{-1}; not shown) but the slow phase rate is again significantly reduced (Fig. 3A). (Note: the fast phase amplitude for this complex may contain a contribution from a decrease in the fluorescence of the intermediate Ca\textsubscript{2}-CaM relative to Ca\textsubscript{4}-CaM-WFF, from the NMR structure of Ca\textsubscript{4}-CaM-M13 (7), the Trp-8 of the WFF might locate between the CaM domains and could be sensitive to the loss of both the N- and C-domain Ca\textsuperscript{2+} ions.)

A similar reduction in slow phase rate is seen in the Quin2-induced dissociation of Ca\textsubscript{4}-CaM-WFF as [peptide]/[CaM] is increased (Fig. 3A). The rate observed with Quin2 is somewhat faster than that for peptide Trp fluorescence (particularly at low [peptide]/[CaM]). This is because a significant amount of Ca\textsubscript{4}-CaM (in equilibrium with Ca\textsubscript{2}-CaM-peptide) is produced in the fast phase (13). In the case of the Quin2 measurements, the fast phase rate also remains unaffected and the fast phase amplitude corresponds to two Ca\textsuperscript{2+} ions at all [peptide]/[CaM] ratios.

In recent work (13) we have studied the dissociation of peptide WF10 (residues 1–10 of WFF) from Ca\textsubscript{4}-CaM-WFF and Ca\textsubscript{4}-TR2C-WF10 (WF10 binds selectively to the C-domain of CaM). In both cases the (monophasic) dissociation rate observed in either peptide-Trp or Quin2 signal decreases as [peptide]/[protein] increases from one to four, also consistent with an analogous relaxation mechanism.

The question then arises of the relative importance of the two alternatives in Fig. 2A, namely the relaxation mechanism (steps 1, 4, and 6) and the intuitively simpler mechanism (steps 1, 5, and 7). Fig. 3, C and D, shows the results of computer
Rate constants used in the simulations shown in Fig. 5
| Rate constant | Path A 5A | Path A 5B | Path A 5C | Path A 5D | Path B 5E | Path B 5F |
|---------------|----------|----------|----------|----------|----------|----------|
| $k_{-1}$      | 30       | 1        | 30       | 30       | 20       | 2        |
| $k_{-2}$      | 1        | 10$^6$   | 1        | 10$^6$   | 10$^6$   | 10$^6$   |
| $k_{-3}$      | 500      | 500      | 2500     | 20       | 700      | 700      |
| $k_{-4}$      | 8.5      | 8.5      | 8.5      | 8.5      | 8.5      | 8.5      |

Numerical Simulation: Effects of Individual Constants on the Overall Kinetics of Disassociation

Two limiting cases, with either path A or B operating in isolation, were simulated using the assumptions outlined under “Materials and Methods.” The rate constants used for the simulations are given in Table III, and the results of the simulations are shown in Figs. 4 and 5. The values of $k_{-1}$ and $k_{-6}$ were fixed at 700 and 8.5 s$^{-1}$, the values measured for CaM alone (Table II). In view of the dominant contribution of pathway 1, 4, 6, pathway 5, 7 is suppressed in these simulations.

Fig. 4 shows the change in species concentrations observed for a typical reaction via path A. Curve f shows the release of Ca$^{2+}$ (monitored as the Quin2 fluorescence change) which is the sum of step 1 (inverse of curve a, disappearance of C$_{NCP}$) plus step 6 (curve d, appearance of C$_{apo-CaM}$). Because of the sequential nature of the pathway, curve d shows a pronounced lag. This is seen most clearly in the signal of Tyr-138 (sensitive to Ca$^{2+}$ dissociation from the C-domain) which can only be monitored with a non-Trp-containing peptide. Because curve d is non-exponential, the summed curve f will also show complex time dependence, but experimentally it will generally appear as a double exponential process. The other normal observable peptide Trp fluorescence is associated with the disappearance of C$_{NCP}$ and C$_{cp}$ (i.e. step 1 plus step 4) and will appear as the inverse of curve e. Again this will be non-exponential owing to the coupling of the two steps plus the fact that step 4 is a reversible relaxation process. The species plot shows the behavior of the intermediate species C$_{cp}$ (curve b) and C$_{cp}$ (curve c) which are involved in this relaxation and also shows how the relaxation rate of step 4 determines the appearance of C$_{cp}$ as step 6 (curve d). In the case of the T26W mutant, where there is a fluorescence change between species C$_{NCP}$ and C$_{cp}$ (step 1) the total Trp fluorescence change (with a Trp-containing peptide) is the sum of curve a plus the inverse of curve e, again analyzing as a double exponential.

Further simulations show the influence of individual rate constants (Table III) on the form of observable signals. The results for path A simulations are shown in Fig. 5, A–D. At low values of $k_{-1}$ all signals are dominated by a single phase with a rate close to $k_{-1}$ (Fig. 5B). At higher values of $k_{-1}$, the Ca$^{2+}$ dissociation signal monitored by Quin2 is clearly biphasic, with approximately equal amplitudes in both phases (Fig. 5, A, C, and D; curve a). Generally, the signals for the Tyr and Trp probes are strongly dependent on the kinetics of the relaxation step (step 4). Except at very low values of $k_{-1}$, the Trp peptide signal is biphasic. The amplitude of the fast phase is dependent on the value of $k_{-4}$, varying from 0.11 to 0.63 for $k_{-4}$ values of 20 to 2500 s$^{-1}$, respectively (Fig. 5, C and D, curve c). The rates of the fast processes for both the Quin2 and the Trp peptide signals are close to $k_{-1}$ at values of $k_{-4}$ ≥ 100 s$^{-1}$ (Fig. 5, A, B, and C). The rates of the slow processes for both the Quin2 and the Trp peptide signals reflect the convolution of the peptide relaxation (step 4) with the C-terminal Ca$^{2+}$ loss (step 6). Thus, the slow rate does not correspond directly to a single step in the mechanism. For this reason the slow rate only changes from 0.5 to 6 s$^{-1}$ as $k_{-4}$ changes from 20 to 2500 s$^{-1}$ (Fig. 5, C and D). The CaM Tyr signal shows a small lag phase and is generally monophasic with a slow rate, except at low $k_{-1}$ where a biphasic signal results from the gradual accumulation of the species C$_{cp}$ (Fig. 5D, curve b). Although the Tyr signal results only from step 5, the rate is slower than 8.5 s$^{-1}$, owing to convolution with the relaxation step (step 4). The T26W Trp signal with a non-Trp peptide is monophasic, with a rate close to $k_{-1}$, directly reflecting the kinetics of step 1. The T26W Trp signal with a Trp peptide basically mirrors the peptide signal and thus also reflects $k_{-1}$ and $k_{-4}$. When the peptide signal is biphasic, the T26W signal has the same rates as the peptide signal but a
shown in Fig. 5, and the results of the simulations are

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peptide relaxation rate (

path is expected to be the dominant dissociation path if the

peptide off-rate, \( k_{-2} \), must also be slower.

A comparison of the simulations of paths A and B indicates

that the Quin2 results are crucial in suggesting which pathway

dominates. Biphasic Quin2 signals indicate path A dissociation

and allow direct determination of the N-terminal Ca\(^{2+}\) dissocia-

tion rate. The T26W results may also be indicative,
especially when the signal observed with a Trp peptide is biphasic.

Path A is indicated if the relative amplitudes of the fast and

slow phases observed with the Trp peptide are reversed when

the peptide is studied with T26W. If the relative amplitudes

remain unchanged, path B is indicated. It is not possible to see

a reversal of the amplitudes if dissociation is occurring via path

B, regardless of the relative fluorescence changes of the two Trp

probes, peptide Trp and T26W. These simulations indicate how

the experimental observables may be expected to deviate from

simple mono- or biphasic exponential kinetics owing to the cou-

pling of the individual steps in both of the pathways A and B.

Interpretation of Observed Kinetics

Results obtained with Quin2 and with the T26W mutant

suggest which dissociation pathway is dominant, but it would

be useful to know the relative magnitudes of \( k_{-2} \) and \( k_{-1} \). We

attempted to measure \( k_{-2} \) directly by displacing Trp-contain-

ing peptides from their Ca\(_4\)-CaM-peptide complexes using a

silent peptide (CBP1 or FFF). The observed rate was strongly

dependent on the concentration of the displacing peptide and

did not reach a limiting value, suggesting an associativeme-

chanism, in which the silent peptide binds to the Ca\(_4\)-

CaM-peptide complex forming a transient intermediate.

WFF Peptide—It is possible to estimate a value for \( k_{-2} \) for a

particular peptide if the dissociation constant, \( K_d \), and the

association rate constant, \( k_{on} \), are known. This value can be

compared with an experimentally determined \( k_{-2} \). As an exam-

ple, consider Ca\(_{2+}\) dissociation from the Ca\(_4\)-CaM-WFF com-

plex (Fig. 1A). The experimentally determined \( k_{on} \) for Ca\(_4\)-CaM

and FWF suggests that a reasonable estimate for \( k_2 \) for the

sk-MLK peptides (20°C) is 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}. The \( K_d \) for WFF

is ~0.12 nM (Table II), so that \( k_{-2} \) is calculated (as \( k_{on}/K_d \)) to be

~0.25 s\(^{-1}\).

For Ca\(_4\)-CaM-WFF, the dissociation process observed

with Quin2 is biphasic (Table II) with a fast phase rate of

~12 s\(^{-1}\), which can be assumed to reflect dissociation of the

N-terminal Ca\(_{2+}\) ions. Since \( k_{-1} \) is very much larger than the

estimated \( k_{-2} \), it is reasonable that the dissociation of Ca\(_4-

CaM-WFF will proceed predominantly via path A. Further

evidence for this conclusion comes from the observation that

the rates for the EGTA-induced dissociation of Ca\(_4\)-CaM-WFF

and Ca\(_4\)/T26W-WFF are similar, but the relative amplitudes

of the fast and slow phases are reversed (Table II). This behavior

resembles the simulated data (Fig. 5D) and is typical of disso-

ciation via path A. The relaxation data (Fig. 3) strongly support

the predominant involvement of path A.

Other Peptides—For peptide complexes with a biphasic

Quin2 signal, it is possible to estimate \( k_{-1} \) in a similar manner,
as the reciprocal of the Quin2-induced dissociation rate.

These values are 31, 65, 14, and 8 s\(^{-1}\) for FWF, MasX, Mas,

and CBP1. Using the peptide affinities listed in Table II and an

estimated maximum \( k_{on} \) of 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}, we obtain

maximum \( k_{-2} \) values of 13, 1.8, 0.6, and 0.01 s\(^{-1}\) for

FWF, MasX, Mas, and CBP1. These values indicate that the peptides

MasX, Mas, and CBP1 are likely to dissociate via path A. The

experimental data obtained for these peptides are consistent with

FIG. 5. Simulated stopped-flow traces for paths A and B. The
rate constants used for the simulations are shown in Table III. Curves
resulting from the formation of Ca\(_{2+}\) monitored by Quin2 fluorescence
(a); apo-CaM (C\(_{ap}\)), monitored by Tyr-138 fluorescence (b); peptide,
monitored by Trp fluorescence (for a Trp peptide) (c); Ca\(_{1}\)-CP, monitored
by T26W Trp fluorescence (for a non-Trp peptide) (d); and peptide plus
C\(_{CP}\), as monitored by peptide and T26W Trp fluorescence (for a Trp
peptide) (e). A: path A, \( k_{-1} = 30 \text{ s}^{-1}, k_{-2} = 500 \text{ s}^{-1} \); B: path A, \( k_{-1} = 1 \text{ s}^{-1}, k_{-2} = 500 \text{ s}^{-1} \); C: path A, \( k_{-1} = 30 \text{ s}^{-1}, k_{-2} = 2500 \text{ s}^{-1} \); D: path A, \( k_{-1} = 30 \text{ s}^{-1}, k_{-2} = 20 \text{ s}^{-1} \); E: path B, \( k_{-2} = 20 \text{ s}^{-1} \); F: path B, \( k_{-2} = 2 \text{ s}^{-1} \).

much greater amplitude in the fast phase (Fig. 5, A and D),
curves e and c). The stability of the species C\(_{ap}\)
determines the
kinetics of the relaxation step and is thus critical in determin-
ing the observed kinetics.

Path B, in which the first step is dissociation of the peptide
from C\(_{NCP}\) (step 2), must be considered as a possibility even
though the initial step does not involve Ca\(_{2+}\) dissociation. This
is because the species C\(_{NCP}\) is in rapid equilibrium with C\(_{NC}\)-
and free peptide. The N-terminal Ca\(_{2+}\) ions will dissociate from
Ca\(_4\)-CaM (C\(_{NC} \) ) at 700 s\(^{-1}\) (Table II) so that loss of Ca\(_{2+}\) (step
3) can compete effectively with peptide recombination (step 2). This
path is expected to be the dominant dissociation path if the
peptide relaxation rate (step 2) is faster than N-terminal Ca\(_{2+}\)
dissociation (step 1). Path B was simulated by varying the
peptide off-rate, \( k_{-2} \), from 1 to 20 s\(^{-1}\), for a fixed peptide
on-rate, \( k_2 \), of \( 10^6 \text{ M}^{-1} \text{ s}^{-1} \), corresponding to affinities of peptide
for Ca\(_4\)-CaM in the range 1 to 20 nM. The rate constants used
are shown in Table III, and the results of the simulations are
shown in Fig. 5, E and F. The Ca\(_{2+}\) dissociation, as monitored
by Quin2, is monophasic and slow (Fig. 5, E and F, curve a).
The signals from any of the Trp probes are identical and consist of
a biphasic trace with a small fast phase (Fig. 5, E and F, curves
\( c, d, \) and e). The fraction of the total amplitude in the fast phase
varies from about 0.15 to 0.3 for \( k_{-2} \) values of 1 to 20 s\(^{-1}\),
respectively. The observed rates do not correlate directly with
single steps of the mechanism and are significantly decreased
by the presence of excess peptide. The simulation is virtually
unaffected if the peptide on-rate, \( k_2 \), is slower than \( 10^6 \text{ M}^{-1} \text{ s}^{-1} \),
because then the peptide off-rate, \( k_{-2} \), must also be slower.

A comparison of the simulations of paths A and B indicates
that the Quin2 results are crucial in suggesting which pathway


 Kinetic Studies of Calmodulin, Peptide Complexes

this pathway. The situation is less clear for the peptide FFW, where \( k_{-1} \) and \( k_{-2} \) may be comparable and paths \( A \) and \( B \) may be in competition. However, the biphasic Quin2 signal, and the reversed relative amplitudes for the biphasic T26W and Trp peptide signals, strongly suggest that \( \text{Ca}_4 \text{-CaM-FFW} \) dissociation also occurs via path \( A \). The experimental curves obtained for FFW (Fig. 1B) closely resemble the simulation of path \( A \) in Fig. 5C, and the relaxation data again strongly suggest the predominant involvement of path \( A \).

The EGTA-induced dissociation of the \( \text{Ca}_4 \text{-CaM-MasX} \) complex, monitored by Trp fluorescence, has a fast phase corresponding to an increase in fluorescence (Fig. 1C). Interestingly, equilibrium fluorescence experiments in which \( \text{Ca}^{2+} \) is titrated into apo-CaM plus MasX show the formation of an intermediate species with high fluorescence at a ratio of 2 mol of \( \text{Ca}^{2+} \) per mol of CaM (29). Evidence for the existence of an intermediate \( \text{Ca}_4 \text{-CaM-peptide} \) species is also seen in \(^1\text{H} \) NMR studies of calmodulin with mastoparan (39). If this intermediate species is a complex in which only the C-terminal \( \text{Ca}^{2+} \) sites are occupied, as might likely be the case, its existence under equilibrium conditions is consistent with the proposed path \( A \) mechanism.

For peptides FFW and FFF, with monophasic Quin2 signals (Table II), it is not possible to say whether path \( A \) or \( B \) dominates. For simulations to even approach the kinetic behavior of these peptides (see Fig. 1B for data on FFF), it was necessary to assume that the N-terminal \( \text{Ca}^{2+} \) dissociation rate, \( k_{\text{-1}} \), was approximately 2 s\(^{-1}\). Since maximum values of \( k_{-2} \) are estimated to be 2 s\(^{-1}\) (FFF) and 3.2 s\(^{-1}\) (FFW), it is highly likely that paths \( A \) and \( B \) operate in competition.

**DISCUSSION**

**The Dissociation Pathway for the Calmodulin-Peptide Complexes**—This work has examined the chelator-induced dissociation of seven \( \text{Ca}_4 \text{-CaM-peptide} \) complexes. A striking finding is that substitution or permutation of residues within the target peptide sequence can have a significant effect not only on the kinetics but also on the overall pathway for the dissociation reactions. For all of the complexes studied, except those with FFF and FFW, we observe biphasic kinetics. This is consistent with a mechanism in which the first event is loss of the N-terminal \( \text{Ca}^{2+} \) ions at a rate which is 10 to 100 times slower than that with CaM alone (700 s\(^{-1}\)). There is little obvious relationship between the rate of this fast process and the affinity of the peptide for \( \text{Ca}_4 \text{-CaM} \). Subsequent dissociation may then occur through loss of the C-terminal \( \text{Ca}^{2+} \) ions, followed by rapid loss of the peptide. The alternative pathway involves peptide loss from the complex in which only the C-terminal \( \text{Ca}^{2+} \) ions are bound, followed by loss of the C-terminal \( \text{Ca}^{2+} \) ions. Coupling of these two steps limits the observed slow phase rate to the range 0.5 to 5 s\(^{-1}\), and there is again no strong correlation with peptide affinity.

By contrast, the \( \text{Ca}_4 \text{-CaM} \) complexes of FFW and FFF show monophasic kinetics with Quin2 and appear to have unexpectedly slow N-terminal \( \text{Ca}^{2+} \) dissociation rates in view of the fact that they bind to \( \text{Ca}_4 \text{-CaM} \) with affinity comparable with that of WFF. The slow N-terminal \( \text{Ca}^{2+} \) dissociation rates suggest that FFW and FFF may have stronger interactions with the N-domain than other peptides. The FFW and FFF peptides have the same sequence as WFF, except that both have a W4F substitution, and FFW has a P17W substitution. All three peptides appear to bind in the same orientation, with residues 4 and 17 interacting with the C- and N-terminal domains of CaM, respectively (11). It is notable that FFF and WFF behave differently, considering that the C-terminal region of both peptides could interact similarly with the N-domain of the CaM. For FFF, the substitution of the anchoring Trp-4 residue may confer a some-

what different conformation on the \( \text{Ca}_4 \text{-CaM-peptide} \) complex. Direct measurement of \( \text{Ca}^{2+} \) binding to apo-CaM plus FFF confirms the enhancement of the N-domain \( \text{Ca}^{2+} \) affinity.

For these M13-related peptides and for CBP1 the rates observed with T26W CaM are similar to, although somewhat faster than, the rates observed with *Drosophila* CaM. For the mastoparans, however, the rates observed with T26W CaM are significantly faster (Table II). This suggests that the interaction of these peptides with calmodulin may have been significantly affected by the mutation in the N-domain, particularly for Mas itself. There is evidence that the mastoparans bind more strongly to the C-domain of CaM than the N-domain (42), and it has been suggested that they bind exclusively to the C-domain in the 1:1 complexes (43). Our results suggest that the mastoparans interact significantly with both domains of CaM, supporting the results obtained with \( \text{Ca}^{2+} \) binding site mutants of CaM (44).

For the chelator-induced dissociation of several CaM-peptide and CaM-protein complexes have been reported. For example, values of 2 and 1 s\(^{-1}\) have been reported for the complexes of CaM with intact sk-MLCK (18) and with the RS-20 peptide from the CaM binding domain of sm-MLCK (19). Rates of 140, 12.1, and 1.1 s\(^{-1}\) have been reported for the Quin2-induced dissociation of CaM-mellitin (45); the two slower rates, which accounted for \(~90\%\) of the total amplitude, were attributed to N- and C-terminal \( \text{Ca}^{2+} \) dissociation, respectively. Snyder et al. (46) report that the binding of an (unspecified) amphipathic peptide reduces the N-terminal dissociation rate by a factor of 150 and the C-terminal rate by a factor of 8. Calmodulin labeled at Cys-27 with the fluorescent probe MI-ANS has been used to study the EGTA-induced dissociation of the CaM complexes with the proteins caldesmon, calponin, and sm-MLCK. The rate determined for the calponin complex (\( K_d = 1 \) nM) was \(~1\) s\(^{-1}\) (21). The rates for the complexes with sm-MLCK (\( K_d = 1.1 \) nM) and caldesmon (\( K_d = 108 \) nM) were 3.5 and 13.5 s\(^{-1}\) (20). The observed kinetics in these systems, namely a slow rate in the range 1–5 s\(^{-1}\) (except for the complex with caldesmon which binds with low affinity), are similar to the results in Table II suggesting that the dissociation mechanism outlined here also be valid for a range of peptides and proteins.

Two new stopped-flow studies have appeared recently. Perschini et al. (23) reported biphasic rates for Quin2-induced dissociation reaction of \( \text{Ca}_4 \text{-CaM-M13} \) of 1.9 and 0.15 s\(^{-1}\) (25 °C). These are approximately 5- and 10-fold slower than we measured for WFF (Table II). However, the corresponding rates for the N- and C-terminally protected WFF peptide are 3.5 and 0.4 s\(^{-1}\). Values of 17.7 and 1 s\(^{-1}\) were reported for dissociation of \( \text{Ca}_4 \text{-CaM-nPEP} \), where nPEP is the target sequence of neuronal nitric oxide synthase. Interestingly, the corresponding experiments with both intact enzymes show fast rates >1000 s\(^{-1}\) (but with different amplitudes). These results indicate that the partially saturated CaM-enzyme complex can exert a significant functional role, which is apparently different in the two systems, being active for sk-MLCK but inactive for neuronal nitric oxide synthase. Johnson et al. (24) also provide evidence for the prior dissociation of the N-domain \( \text{Ca}^{2+} \) ions from CaM complexes with target peptides (from CaM-dependent protein kinase II, peptides RS20 and M13 from sm- and sk-MLCK, and MARCKS peptide) and deduced that the corresponding intermediate partially saturated CaM-enzyme species could function in a manner similar to the action of troponin C on skeletal muscle.

**The Role of the Intermediate Species, \( \text{Ca}_4 \text{-CaM-Peptide} \)—A second striking feature is the important role of the intermediate species \( \text{Ca}_4 \text{-CaM-peptide} \) with two \( \text{Ca}^{2+} \) ions in the C-domain. Numerical simulations show that a potentially impor-
tant factor in determining the observed dissociation kinetics is the affinity of the peptide in this intermediate. If we assume that the increase in Ca$^{2+}$ affinity for CaM on peptide binding is fully reflected in changes in the Ca$^{2+}$ dissociation rates, the extent to which Ca$^{2+}$ binding to each of the N-terminal sites is enhanced by the peptide is calculated as $700/k_{off}$, where $k_{off}$ is the estimated dissociation rate for the N-terminal sites in the presence of the peptide. The factor by which peptide binding is enhanced by binding of the N-terminal Ca$^{2+}$ ions is then $(700/k_{off})^2$, and the value of the dissociation constant for interaction of the peptide with the Ca$^{2+}$-CaM species is $K_d(\text{INT}) = K_d(700/k_{off})^2$. Using the values measured for the fast phase of Quin2-induced dissociation as our estimate of $k_{off}$, we calculate values of $K_d(\text{INT})$ of 600 nM (WFF), 3.3 μM (FWF), 100 nM (MasX), 700 nM (Mas), and 40 nM (CBP1) (Note: For FFW and FFF we estimate values of 100 and 60 μM using an upper limit for $k_{off}$ of 4 s$^{-1}$). The $K_d(\text{INT})$ values calculated for MasX and WFF are consistent with values measured for dissociation constants of these peptides interacting with TR2C, the C-terminal tryptic fragment of CaM, of $<200$ nm for MasX (42), and of $\sim700$ nm for WFF (13).

These values of $K_d(\text{INT})$ are consistent with the observations that the species Ca$^{2+}$-CaM-peptide is an important intermediate in the major pathway for the response of the complex to removal of Ca$^{2+}$. The existence of the relaxation step also suggests a mechanism for regulating the CaM-target sequence interaction in terms of the fast reversible exchange between the species Ca$^{2+}$-CaM-peptide and Ca$^{2+}$-CaM. This is in contrast to the obligatory removal of Ca$^{2+}$ ions from the C-domain, as implied by the step 5/step 7 mechanism. This exchange reaction could be important in the regulation of the typical CaM-enzyme system where, owing to the proximity of the target sequence, the effective concentration of the target is enhanced (47), hence stabilizing the intermediate complex. This species could then serve to ensure attachment of CaM to the target protein via the C-domain, with enzyme activation requiring binding of Ca$^{2+}$ to the N-domain to enable its interaction with the target (12, 13). It is notable that this mechanism would be possible for target sequences which followed path A (rather than B), a distinction which is evidently conferred at least in part by the characteristics of the individual target sequences.

In conclusion, this study shows that the dissociation reactions of Ca$^{2+}$-CaM-peptide complexes in the presence of Ca$^{2+}$-chelators are perhaps more complicated than has previously been appreciated, but they are nonetheless capable of being resolved into a number of specific components. Irrespective of the detailed molecular mechanism, it is clear that, in general, observed dissociation rate constants are related only indirectly to the kinetics of individual steps in the Ca$^{2+}$ dissociation processes. For the peptides studied, the predominant dissociation path appears to be loss of the N-terminal Ca$^{2+}$ ions followed by loss of the peptide in a relaxation process and is indicated by biphasic Quin2 and EGTA kinetics. An alternative dissociation path involving initial loss of the peptide may also be important for some peptides. The results indicate that subtle changes in the peptide sequence can have significant effects on the dissociation kinetics and therefore on the relative importance of different pathways. Extrapolating to the biological function of CaM with target proteins, this diversity suggests that different intermediate states such as Ca$^{2+}$-CaM-peptide may be generated which can modulate either enzymatic activity or kinetic properties related to the specific regulatory processes involved, in a way which is highly dependent on the specific target sequence.