Different Mechanisms for Ca2+ Dissociation from Complexes of Calmodulin with Nitric Oxide Synthase or Myosin Light Chain Kinase*

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We have determined the stoichiometry and rate constants for the dissociation of Ca2+ ion from calmodulin (CaM) complexed with rabbit skeletal muscle myosin light chain kinase (skMLCK), rat brain nitric oxide synthase (nNOS), or with the respective peptides (skPEP and nPEP) representing the CaM-binding domains in these enzymes. Ca2+ dissociation kinetics determined by stopped-flow fluorescence using the Ca2+ chelator quin-2 MF are as follows: 1) Two sites in the CaM-nNOS and CaM-nPEP complexes have a rate constant of 1 s⁻¹. 2) The remaining two sites have a rate constant of 18 s⁻¹ for CaM-nPEP and >1000 s⁻¹ for CaM-nNOS. 3) Three sites have a rate constant of 1.6 s⁻¹ for CaM-skMLCK and 0.15 s⁻¹ for CaM-skPEP. 4) The remaining site has a rate constant of 2 s⁻¹ for CaM-skPEP and >1000 s⁻¹ for CaM-skMLCK. Comparison of these rate constants with those determined for complexes between the peptides and tryptic fragments representing the C- or N-terminal lobes of CaM indicate a mechanism for Ca2+ dissociation from CaM-nNOS of 2C slow + 2N fast and from CaM-skMLCK of (2C + 1N) slow + 1N fast. Ca2+ removal inactivates CaM-nNOS and CaM-skMLCK activities with respective rate constants of >10 s⁻¹ and 1 s⁻¹. CaM-nNOS inactivation is fit by a model in which rapid Ca2+ dissociation from the N-terminal lobe of CaM is coupled to enzyme inactivation and slower Ca2+ dissociation from the C-terminal lobe is coupled to dissociation of the CaM-nNOS complex. CaM-skMLCK inactivation is fit by a model in which the three slowly dissociating Ca2+ binding sites are coupled to both dissociation of the complex and enzyme inactivation.

An understanding of cellular regulation will require elucidation of the different kinetic mechanisms by which CaM regulates the many enzyme activities under its control. In this respect it is clear that different structural domains in CaM contribute quite variably to both the activation and binding of different target enzymes (George et al., 1990; Persechini et al., 1994). For example, it was recently demonstrated that fragments representing the N- and C-terminal lobes of CaM, each of which contains one of the two pairs of EF-hand Ca2+-binding sites in CaM, bind to their respective sites on neural nitric oxide synthase (nNOS) and skeletal muscle myosin light chain kinase (skMLCK) with affinities differing by a factor of 100 or more between the two enzymes, although the concentrations of intact CaM required for half-maximal activation of these enzyme activities are not significantly different (Persechini et al., 1994).

Olwin et al. (1984) and Olwin and Storm (1985) have demonstrated free energy coupling between target and Ca2+ binding to CaM such that the Ca2+-binding affinity of CaM in its complex with the target is increased over what is seen with free CaM in proportion to the relative increase in target binding affinity caused by Ca2+ binding to CaM. Our recent results with CaM tryptic fragments suggest that the free energy coupling associated with Ca2+-dependent binding of CaM to nNOS and skMLCK is not equally distributed among the four Ca2+-binding sites of CaM. As a result, the roles of the two CaM lobes in establishing Ca2+-dissociation and enzyme inactivation rates for the CaM-nNOS and CaM-skMLCK complexes should differ significantly.

In this study we have investigated the rates of Ca2+ dissociation from the CaM-nNOS and CaM-skMLCK complexes and the rates of enzyme inactivation associated with this process. We have confirmed substantial differences between the mechanisms of Ca2+ dissociation from the two CaM enzyme complexes that are attributable to the distinct roles played by the CaM lobes in these complexes.

**MATERIALS AND METHODS**

Vertebrate CaM was expressed in Escherichia coli and purified as described by Persechini et al. (1989). skMLCK was expressed in Sf9 cells and purified as described by Fitzsimmons et al. (1992). nNOS was expressed in HEK A293 cells and purified essentially as described by McMillan et al. (1992). CaM tryptic fragments TRCl and TRCII were generated and purified as described by Persechini et al. (1994). Synthetic peptides; KRAIGFKLAEVKFSAKLMGQ-amide (nPEP), based on the reported CaM-binding domain in nNOS (Vorher et al., 1993; Zhang and Vogel, 1994), and KRRWKKNFIAVSANRFKK-amide (skPEP), based on the reported CaM-binding domain in skMLCK (Blumenthal and Krebs, 1987), were commercially synthesized (Quality Controlled Biochemicals, Inc.) and were verified by mass spectrometry.

Enzyme inactivation experiments were performed at 25°C with a pneumatically driven three syringe mixing device built and used as described by McCarthy et al. (1994). Reactions containing 1 μM CaM and 650 nM nNOS or 50 nM skMLCK were initiated by adding L-[3,4,5-3H]arginine (50,000 cpm/pmol; Amersham Corp.) or 32P-ATP (1000 cpm/pmol; DuPont NEN) to final concentrations of 1.4 μM or 1 mM, respectively. The reaction buffer for both enzyme reactions contained 50 mM MOPS, 150 mM NaCl, 1 mM dithiothreitol, 200 μM CaCl₂, and 10 mM...
MgCl₂, pH 7.0. For nNOS reactions the buffer also contained 1 μM tetrahydrobiopterin and 100 μM NADPH. Reactions were quenched at the indicated times by addition of 1 volume of either 150 mM phosphoric acid (skMLCK) or 15% trichloroacetic acid (nNOS). Aliquots of the quenched samples were processed to quantitate amounts of product as described previously (Persechini et al., 1994). For measurements of enzyme inactivation rates, samples were quenched at various times after addition of EGTA to a final concentration of 3 mM. Theoretical curves for enzyme inactivation were generated using an integrated rate equation where P is the amount of product, V is the linear enzyme rate prior to EGTA addition, k₁ is the first-order rate of inactivation after EGTA addition, t is the time after addition of the chelator, and C is the amount of product present when EGTA is added to the reaction mixture.

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P = \frac{Ve^{k_1 t} + V}{k_1 + C} \quad \text{(Eq. 1)}
\]

Curve fitting to data for enzyme inactivation time courses was performed using the Prism software package (GraphPad, Inc.)

Presteady-state measurements of changes in quin-2 MF (Molecular Probes) fluorescence observed at 90° to the excitation beam were performed using a stopped-flow fluorimeter with a dead time of 1.5 ms (Applied Photophysics, Leatherhead, United Kingdom). The quin-2 MF excitation wavelength at 320 nm was obtained using a 0.124-m monochromator (Farrand Corp.) illuminated by a 100-watt mercury arc lamp. Fluorescence emission from quin-2 MF was isolated with a 520 nm high-pass glass filter (Corion). Four or more stopped-flow time courses of 1024 data points were averaged for each experiment. Data were filtered with a time constant of 0.4 ms and collected with a Nicolet Explorer III digital oscilloscope and finally transferred to a Zenith HL-14B PC for permanent storage and analysis. Observed rate constants were obtained by fitting the data to single or double exponential equations by the method of moments (Dyson and Isenberg, 1971) or using the Prism software package (GraphPad, Inc.). Stopped-flow experiments were performed at 25 °C.

For measurements of Ca²⁺ dissociation from CaM-nNOS or CaM-skMLCK complexes, syringes A of the stopped-flow apparatus contained 2 μM CaM, a molar excess of enzyme over CaM, and 50 μM CaCl₂. Enzyme was omitted for measurements of Ca²⁺ dissociation from CaM alone. Syringe B contained 200 μM quin-2 MF. Both syringes contained 150 mM NaCl, 20 mM MOPS, 0.5 mM dithiothreitol, pH 7.0. Similar conditions were used for measurements of Ca²⁺ dissociation from complexes between nPEP or skPEP and CaM, TRCI, or TRCII except that the concentration of CaM or CaM fragment in syringe A was 6 or 5 μM, respectively. Measurements were made at 2 or more peptide/CaM stoichiometries. Measurements of the decay in intrinsic Trp fluorescence of the CaM-skPEP complex were made under conditions identical to those described above, except the excitation wavelength was 295 nm and fluorescence emission from Trp was isolated with a 320–380 nm band-pass glass filter (Oriel Corp.), and Ca²⁺ was chelated with EDTA at a final concentration of 1 mM instead of with quin-2 MF. In all cases reactions were initiated by mixing equal volumes from syringe A and B so that the final concentrations of protein, CaCl₂, and quin-2 MF were half of their initial values.

Calibration of the photomultiplier voltage with respect to increases in Ca²⁺ was performed by mixing a set of CaCl₂ standards with buffer containing the quin-2 MF indicator. This indicator was chosen in preference to quin-2 because of its 5-fold higher affinity for Ca²⁺ ion (Kd = 25 μM; Haughland (1992)). The dependence of the photomultiplier voltage upon added free Ca²⁺ was found to be linear with a slope of 57 mV/μM added CaCl₂. This slope was used to determine the stoichiometry of Ca²⁺ release from CaM, TRCI, and TRCII. A reference for Ca²⁺-free indicator obtained by mixing the indicator with 10 μM EDTA provides an estimate of free Ca²⁺ in the buffer of 2 μM. Quin-2 has been reported to bind Ca²⁺ ion with an association rate constant of 7.5 × 10² M⁻¹ s⁻¹, so at the final concentration of 100 μM used in stopped-flow experiments the rate of Ca²⁺ binding to quin-2 would exceed any measurable Ca²⁺ dissociation rate by at least a factor of 100 (Bayley et al., 1984).

150 mM NaCl was added to experimental buffers to prevent Ca²⁺- independent interactions between CaM and peptides, which have been reported to occur at lower ionic strengths (Itakura and Iio, 1992). We have found that interactions between CaM and a peptide based on the CaM-binding domain in skMLCK are not completely reversed by Ca²⁺ chelation unless 150 mM NaCl is present. Salt at this concentration does not significantly affect the ability of CaM to activate nNOS or skMLCK activities (data not shown). The rate constant for EGTA-induced inactivation of skMLCK activity we report here (1.0 s⁻¹) is not significantly different from the value of 0.8 s⁻¹ determined by Stull et al. (1985) in the absence of added NaCl.

RESULTS

Data for ³²P incorporation into peptide substrate by skMLCK are presented in Fig. 1. The time course for this reaction is linear over the experimental interval of 5 s (Fig. 1, inset). Chelation of Ca²⁺ ion by addition of EGTA 3 s after initiation of the phosphorylation reaction causes a relatively slow inactivation of skMLCK activity (Fig. 1, inset). Fitting data collected during the first 2 s of this inactivation phase gives an inactivation rate constant of 1 s⁻¹ (Fig. 1). As seen with skMLCK activity, nNOS activity is linear over the experimental time interval; however, EGTA addition causes a more rapid inactivation that is too fast to measure. As seen in Fig. 2, an inactivation rate constant of 1 s⁻¹ gives a poor fit to the data. A rate constant of 10 s⁻¹ also appears too slow to describe the inacti-
Data for dissociation of Ca\textsuperscript{2+} ion from the CaM-skMLCK complex are presented in Fig. 3A. The data are fit well by single exponentials with respective rate constants of 1.6 and 1 s\textsuperscript{-1}. However, the amplitudes of the signals for the two CaM-enzyme complexes are clearly different. For the CaM-skMLCK complex, an amplitude of 2.8 mol of Ca\textsuperscript{2+}/mol of CaM is obtained; the corresponding amplitude for the CaM-nNOS complex is 1.9 mol of Ca\textsuperscript{2+}/mol of CaM. Data for Ca\textsuperscript{2+} release from free CaM are fit by a single exponential with a rate constant of 12.6 s\textsuperscript{-1} and an amplitude of 1.9 mol of Ca\textsuperscript{2+}/mol of CaM.

In contrast with free CaM or the CaM-enzyme complexes, we were able to observe Ca\textsuperscript{2+} dissociation from all four Ca\textsuperscript{2+}-binding sites in the CaM-peptide complexes. Data for Ca\textsuperscript{2+} release from the CaM-nPEP and CaM-skPEP complexes are presented in Fig. 3B. Data for only the first 3 s are shown so that the fast phase of Ca\textsuperscript{2+} release from CaM-nPEP can be seen. Data for Ca\textsuperscript{2+} release from CaM-nPEP are fit by a double exponential with rate constants of 1 and 17.7 s\textsuperscript{-1}, and amplitudes of 1.8 and 2.1 mol of Ca\textsuperscript{2+}/mol of CaM, respectively (Fig. 3B). Data for CaM-skPEP are fit by a double exponential with rate constants of 0.15 and 1.9 s\textsuperscript{-1} and amplitudes of 2.8 and 0.9 mol of Ca\textsuperscript{2+}/mol of CaM, respectively (Fig. 3B).

In an attempt to match observed Ca\textsuperscript{2+} dissociation rates with one or the other of the two CaM lobes, we investigated the kinetics of Ca\textsuperscript{2+} release from TRCI or TRCII either alone or in the presence of a molar excess of nPEP or skPEP. Data for dissociation of Ca\textsuperscript{2+} ion from free TRCII are fit by a single exponential with a rate constant of 16 s\textsuperscript{-1} and an amplitude of 2 mol of Ca\textsuperscript{2+}/mol of TRCII; Ca\textsuperscript{2+} release from free TRCI is too fast to measure. In the presence of a molar excess of skPEP, Ca\textsuperscript{2+} release from TRCII is described by a single exponential with a rate constant of 0.34 s\textsuperscript{-1} and an amplitude of 2 mol of Ca\textsuperscript{2+}/mol of TRCII. Under these conditions, release of Ca\textsuperscript{2+} ion from the TRCI is described by a single exponential with a rate constant of 4.3 s\textsuperscript{-1} and an amplitude 2.1 mol of Ca\textsuperscript{2+}/mol of TRCI (Fig. 4). In the presence of a molar excess of nPEP, Ca\textsuperscript{2+} release from TRCII is described by a single exponential with a rate constant of 1.1 s\textsuperscript{-1} and an amplitude of 2.2 mol of Ca\textsuperscript{2+}/mol of TRCII. Release of Ca\textsuperscript{2+} ion from the TRCI under these conditions is described by a single exponential with a rate constant of 100 s\textsuperscript{-1} and an amplitude of 1.8 mol of Ca\textsuperscript{2+}/mol of TRCI (Fig. 4).

It is well established that on binding CaM the single Trp residue in skPEP undergoes a fluorescence enhancement and its emission wavelength is blue-shifted due to its interaction with the C-terminal lobe in CaM (Blumenthal and Krebs, 1987; Ikura et al., 1992). We therefore examined the rate of decrease in Trp fluorescence seen during Ca\textsuperscript{2+} release from the CaM-skPEP complex. We found this process to be described by a single exponential with a rate constant of 0.22 s\textsuperscript{-1} (data not shown).

**DISCUSSION**

We have found that, when CaM is complexed with skMLCK, the apparent Ca\textsuperscript{2+} dissociation rate constant for two sites in CaM is reduced from 12.6 to 1.6 s\textsuperscript{-1} and the rate constant for 1 site is reduced from > 1000 s\textsuperscript{-1} to 1.6 s\textsuperscript{-1}. With the CaM-nNOS complex, there is a similar reduction in the Ca\textsuperscript{2+} dissociation rate of the two more slowly dissociating sites (Table I). Our results indicate that the kinetic mechanisms for Ca\textsuperscript{2+} release from the CaM-enzyme complexes are established by interactions present in both the CaM-enzyme and CaM-peptide complexes. Hence, the CaM-peptide complexes provide models for elucidating the structural basis for the distinct mechanisms of Ca\textsuperscript{2+} release followed by CaM-nNOS and CaM-skMLCK.

Kasturi et al. (1993) have determined the rate of Ca\textsuperscript{2+} dissociation from the complex between wheat germ CaM and avian gizzard myosin light chain kinase using a quin-2 indicator. These investigators have reported that the rate of Ca\textsuperscript{2+}...
release from free wheat germ CaM is decreased 5-fold when it is bound to the gizzard kinase; a similar decrease was reported to occur when CaM is complexed with mellitin (Kasturi et al., 1993). Although no attempt to determine Ca\(^{2+}\) release stoichiometries was made, it was reported that the amplitude for the observed quin-2 fluorescence transient is increased 2-fold when CaM is complexed with gizzard myosin light chain kinase (Kasturi et al., 1993). These observations suggest that the properties of the CaM-gizzard myosin light chain kinase complex with respect to Ca\(^{2+}\) release and inactivation are likely to be similar to what we report here for the CaM-skMLCK complex.

In order to locate the observed Ca\(^{2+}\)-binding sites in CaM-nNOS or CaM-skMLCK in one or the other of the two CaM lobes, we determined Ca\(^{2+}\) dissociation rates for the complexes between TRC1 or TRCII and either of skPEP or nPEP. We used the CaM-peptide complexes for these determinations because they follow kinetic patterns similar to those seen with the CaM-enzyme complexes (3 slow + 1 fast for CaM-skMLCK and 2 slow + 2 fast for CaM-nNOS), but slower dissociation rates allow all four Ca\(^{2+}\)-binding sites in CaM to be observed. Dis-

**Fig. 3. Release of Ca\(^{2+}\) ion from CaM-enzyme or CaM-peptide complexes.** Quin-2 MF fluorescence data were converted to Ca\(^{2+}\) release stoichiometries using a ratio of 57 mV/\(\mu\)M Ca\(^{2+}\) determined as described under “Materials and Methods.” The final quin-2 MF concentration was 100 \(\mu\)M. Panel A contains data for Ca\(^{2+}\) release from CaM-skMLCK and CaM-nNOS after addition of quin-2 MF. The final CaM concentration was 1 \(\mu\)M, with final skMLCK and nNOS concentrations of 1.3 \(\mu\)M and 1.9 \(\mu\)M, respectively. Panel B contains data for Ca\(^{2+}\) release from CaM-nPEP and CaM-skPEP after addition of quin-2 MF. The final CaM concentration was 3 \(\mu\)M with final skPEP and nPEP concentrations of 5 \(\mu\)M. Curves are single or double exponentials calculated using the rate constants and amplitudes given in Table I.
association of 2 Ca$^{2+}$ ions from the TRCII-nPEP complex occurs with an apparent rate constant essentially identical to the slower Ca$^{2+}$ dissociation rate constant for the CaM-nPEP complex (Table I). Two Ca$^{2+}$ ions dissociate from the TRCII-nPEP complex with an apparent rate 5-fold faster than the faster rate for the CaM-nPEP complex (Table I). These observations indicate that the more slowly dissociating pair of sites in the CaM-nPEP complex is located in the C-terminal CaM lobe. Analysis of the CaM-skPEP complex is complicated by the observation that 3 Ca$^{2+}$ ions dissociate with a single apparent rate: 2 from one CaM lobe and 1 from another (Table I). Ca$^{2+}$ dissociation from the TRCII-skPEP complex occurs with a rate 2-fold faster than the slower rate for the CaM-skPEP complex and 5-fold slower than the faster dissociation rate for this complex (Table I). Ca$^{2+}$ dissociates from the TRCII-skPEP complex twice as fast as the faster rate for the CaM-skPEP complex and 10-fold faster than the slower dissociation rate for this complex (Table I). This suggests that two of the three more slowly dissociating Ca$^{2+}$-binding sites in the CaM-skPEP or CaM-skMLCK complexes are located in the C-terminal lobe of CaM. Similarly to what we report here, Suko et al. (1986) and Itakura and Iio (1992) have reported that CaM
binding to mellitin reduces the Ca$^{2+}$ dissociation rates for all four sites in CaM, with the more slowly dissociating sites localized in the C-terminal lobe of CaM.

Inactivation of the CaM-dependent skMLCK activity upon removal of Ca$^{2+}$ ion occurs with a rate constant of 1 s$^{-1}$; inactivation of CaM-dependent nNOS activity is too fast to measure ($>10$ s$^{-1}$). Dissociation of the CaM-skMLCK complex has been reported to occur with an apparent rate constant of 2 s$^{-1}$ (Johnson et al., 1981), and kinetic measurements of intrinsic tryptophan fluorescence suggest that the CaM-skPEP complex dissociates at a rate of 0.22 s$^{-1}$, close to the slow rate for Ca$^{2+}$ release from this complex (Table I). These observations suggest that slow release of 3 Ca$^{2+}$ ions from the two CaM lobes in a concerted manner is coupled to both inactivation and dissociation of the CaM-skMLCK complex. Inactivation of CaM-nNOS catalytic activity occurs at least 10-fold faster than the rate of Ca$^{2+}$ dissociation from the two sites in the C-terminal lobe of CaM in this complex. This suggests that Ca$^{2+}$ dissociation from the N-terminal lobe of CaM is coupled to inactivation of nNOS activity, while dissociation of Ca$^{2+}$ from the C-terminal lobe is coupled to dissociation of the CaM-nNOS complex. This is consistent with the apparent dissociation constant of 10 nm for the (Ca$^{2+}$)$_n$TRCII-nNOS complex (Perschini et al., 1994). Given a probable association rate of 10$^7$ M$^{-1}$ s$^{-1}$ (Bowman et al., 1992; Meyer et al., 1992; Kasturi et al., 1993), when saturated with Ca$^{2+}$ ion the C-terminal lobe in CaM would be expected to dissociate from nNOS with a rate constant of 0.1 s$^{-1}$, 10-fold slower than the rate constant for Ca$^{2+}$ release from this lobe.

This study demonstrates substantial differences between the CaM-skMLCK and CaM-nNOS complexes with respect to the kinetic mechanisms for Ca$^{2+}$ dissociation and associated inactivation of enzyme activity. These differences reflect the different contributions of the two CaM lobes to the stabilities and catalytic activities of these complexes (Perschini et al., 1994).

A surprising aspect to these results is the observation that 3 Ca$^{2+}$ ions dissociate from the CaM-skMLCK complex in a concerted manner. This may be a reflection of interactions between the CaM lobes that are evident in the complex between CaM and a peptide based on the CaM-binding domain in skMLCK (Ikura et al., 1992). Alternatively, it may be due to slowing of the dissociation rate for one Ca$^{2+}$ from the N-terminal lobe due to specific interactions between this lobe and the peptide sequence. The differences between the CaM-skMLCK and CaM-nNOS complexes we report here indicate that these enzyme activities have distinct spatio-temporal distributions in response to waves in the intracellular Ca$^{2+}$ ion concentration. This is an important dimension to our understanding of CaM function, further illuminating how this seemingly simple protein might shape complex and subtle cellular responses.

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