Calcium-Induced Sensitization of the Central Helix of Calmodulin to Proteolysis

Julia Mackall[‡] and Claude B. Klee*

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received November 20, 1990; Revised Manuscript Received March 12, 1991

ABSTRACT: The rate of proteolysis of trypsin-sensitive bonds was used to examine the nature of the structural changes accompanying Ca^{2+} and Mg^{2+} binding to calmodulin. In the Ca^{2+}-free form, the rates of proteolysis at Arg-106 and Arg-37 are rapid (>300 and 28 nmol min^{-1} mL^{-1}, respectively), the bonds at Arg-74, Lys-75, and Lys-77, in the central helix, are cleaved more slowly (10 nmol min^{-1} mL^{-1}), and a lag in the cleavage at the remaining bonds (Lys-13, Lys-30, Arg-86, Arg-90, and Arg-126) suggests that they are not cleaved in the native protein. High concentrations of Ca^{2+}, but not Mg^{2+}, almost completely abolish proteolysis at Arg-106 and drastically reduce the rate of cleavage at Arg-37. Both Ca^{2+} and Mg^{2+} exert a moderate protective effect on the proteolysis of the central helix. These results suggest that the F-helix of domains III and, to a lesser extent, the F-helix of domain I are somewhat flexible in the Ca^{2+}-free form and are stabilized by Ca^{2+}. Whereas full occupancy of the four Ca^{2+}-binding sites produces little change in the susceptibility of the central helix to proteolytic attack, binding of two Ca^{2+} produces a 10-fold enhancement of the rate of proteolysis in this part of the molecule. We propose that at intermediate Ca^{2+} levels the flexibility of the central helix of calmodulin is greatly increased, resulting in the transient formation of intermediates which have not been detected by spectroscopic techniques but are trapped by the irreversible action of trypsin.

The structural changes that accompany Ca^{2+} binding to calmodulin and are responsible for its dramatic increase in affinity for target proteins are not yet fully understood. The structure of Ca^{2+}-saturated calmodulin, crystallized at pH 5 to 6, has been determined at 2.2-Å resolution (Babu et al., 1988). In the crystal, fully liganded calmodulin consists of two globular lobes, connected by a long central helical linker with a small bend or flexible area in its center (Babu et al., 1985; Kretsinger et al., 1986). Within each lobe, the two adjacent Ca^{2+}-binding domains interact with each other (Dalgarno et al., 1984; Ikura et al., 1985; Tsalkova & Privalov, 1985) and bind Ca^{2+} cooperatively (Minowa & Yagi, 1984). Each lobe also contains a hydrophobic patch believed to serve as a binding site for calmodulin targets (LaPorte et al., 1980; Tanaka & Hidaka, 1980; Vogel et al., 1983; Newton et al., 1984). The size and shape of the globular domains determined by small-angle X-ray scattering studies of calmodulin in solution are consistent with those in the crystal, but the distance between them is significantly shorter (Seaton et al., 1985; Heidorn & Trewhella, 1988; Yoshino et al., 1989). The structure of Ca^{2+}-saturated calmodulin in solution, currently being determined by three-dimensional nuclear magnetic resonance (NMR),[1] may help to explain this discrepancy.[2]

Very little is known about the structure of Ca^{2+}-free calmodulin except that it is different from that of Ca^{2+}-saturated calmodulin [for reviews, see Forsen et al. (1986), Evans et al. (1988), and Klee (1988)]. Attempts to crystallize calmodulin in the absence of Ca^{2+} have been unsuccessful, and NMR spectra suitable for structure determination have not yet been obtained. Small-angle scattering experiments indicate that Ca^{2+}-free calmodulin is less extended than is the Ca^{2+}-saturated form (Seaton et al., 1985; Heidorn & Trewhella, 1988; Yoshino et al., 1989), and circular dichroism studies indicate that the Ca^{2+}-free calmodulin is less helical (Klee & Vanaman, 1982; Hennessey et al., 1987). Fourier-transformed infrared spectra, however, failed to confirm the Ca^{2+}-induced increase in helicity and were interpreted to suggest that a regular α-helix is converted to a distorted helix upon Ca^{2+} binding (Trewhella et al., 1989).

To reconcile the small-angle scattering results with the crystallographic data, Heidorn and Trewhella (1988) proposed a "bent" model for Ca^{2+}-saturated calmodulin in solution. A bend in the middle of the central helix has also been proposed to explain the following: the stimulation of myosin light chain kinase by a calmodulin derivative in which the N- and C-domains[3] are cross-linked and by calmodulin mutants lacking one, two, or four residues in the central linker region (Persechini & Kretsinger, 1988; Persechini et al., 1988); the interaction of norchlorpromazine covalently attached to Lys-75 with both the N- and C-domains of calmodulin (Newton & Klee, 1989); the labeling of Met-71 and -144 with a 23-residue photoreactive calmodulin-binding peptide (O'Neil et al., 1989); the NMR spectra and small-angle X-ray scattering measurements of calmodulin complexed with mastoporan or the calmodulin-binding domain of myosin light chain kinase (Heidorn et al., 1989; Kataoka et al., 1989; Yoshino et al., 1989; Ikura et al., 1989). Flexibility in the central helix could therefore be essential for the mechanism of action of calmodulin. An important functional role for the central helix was suggested by the 100-1000-fold decrease in affinity of calmodulin for its targets following cleavage of the molecule in the middle of the central helix (Kuznicki et asl., 1981; Newton et al., 1984) despite the fact that the resulting fragments have apparently preserved their native structure (Dalgarno et al., 1984; Ikura et al., 1984; Aulabaugh et al., 1984; Klevit et al., 1984). Modifications in the central helix have been shown

* Author to whom correspondence should be addressed.

‡ Present address: Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.

[1] Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl chloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

[2] M. Ikura and A. Bax, personal communication.

[3] The N- and C-domains designate the amino (residues 1-74) and carboxyl (residues 85-148) domains of calmodulin.

to markedly and differentially affect its ability to interact with and activate different enzymes (Putkey et al., 1988; Persechini et al., 1989; Van Berkum et al., 1990).

In this paper, we monitor local Ca^{2+}-induced conformational transitions by measuring the rates of proteolysis at individual bonds throughout calmodulin. Four specific regions of the calmodulin molecule are monitored: the Ca^{2+}-binding domain I, the C-terminal domain consisting of Ca^{2+}-binding domains III and IV, and the carboxyl terminus of the F-helix of Ca^{2+}-binding domain II (central helix). These domains differ significantly both in susceptibility to trypsin and in their responses to Ca^{2+}. We report the observation of a 10-fold enhancement of the rate of proteolysis at bonds located within the central helix upon partial, but not complete, occupancy of the Ca^{2+} sites and propose that at intermediate Ca^{2+} levels calmodulin exists as a novel conformer.

Materials and Methods

Materials. Calmodulin was purified from bovine testis by the method of Newton et al. (1988). The sources of other reagents were as described previously (Newton et al., 1984).

Proteolysis. Limited proteolysis by trypsin was performed by a modification of the method of Newton et al. (1984). Calmodulin (5 mg/mL) in 0.05 M $(NH_4)_2HCO_3$/0.05 M NaCl was incubated with 20 μg/mL trypsin at 30 °C. Proteolysis experiments were performed at various Ca^{2+} concentrations as described under Results. When present, EGTA concentration was 2 mM to completely eliminate calmodulin binding of Ca^{2+} and other metal ions. Digestion of calmodulin was also measured at 1 mM Mg^{2+}. In the latter case, EGTA (0.1 mM) was included in both the presence and the absence of Mg^{2+} to eliminate the possibility of interference by contaminating Ca^{2+}. At the times indicated, aliquots were removed, and trypsin digestion was stopped with 50 μg/mL soybean trypsin inhibitor. Digests were stored at −70 °C prior to chromatography.

Analysis of Trypsin Digests by HPLC. High-pressure liquid chromatography was carried out as described (Newton et al., 1984; Manalan & Klee, 1987; Newton & Klee, 1989). Peptides were identified from previously determined retention times and when needed by amino acid composition or sequence analysis. Since few of the tryptic peptides absorb at 280 nm (calmodulin contains no tryptophan residues and only two tyrosyl residues at positions 99 and 138), elution was monitored at both 215 and 280 nm to facilitate identification of the fragments.

Determination of Proteolytic Cleavage at Arg-74, Lys-75, and Lys-77. Individual rates of cleavage at Arg-74, Lys-75, and Lys-77 were determined by analysis of amino-terminal residues produced during proteolysis according to the procedure of Weiner et al. (1972). Alanine (50 μM) was added to proteolysis reaction mixtures for standardization of recovery. After incubation with trypsin for 10, 20, and 30 min, 10-μL aliquots were removed and added to 40 μL of 0.25 M $NaHCO_3$ containing diisopropyl fluorophosphate (0.0285 M) and $CaCl_2$, $MgCl_2$, and EGTA to produce final concentrations of 0.5 mM Ca^{2+}, 0.25 mM Mg^{2+}, and 0.5 mM EGTA and lyophilized 3 times to remove NH_4HCO_3. Twenty microliters of 0.2 M $NaHCO_3$, pH 9.8, was added prior to the last lyophilization. The dry samples were dissolved in 20 μL of H_2O and incubated with 20 μL of 12.5 mM [^{14}C]dansyl chloride (35 000 cpm/nmol) in acetone for 120 min at 37 °C in the dark. Samples were hydrolyzed for 19 h at 110 °C in 6 N HCl. HCl was removed by lyophilization, and samples were dissolved in 5 μL of 50% pyridine. A mixture containing the unknown (0.5 μL), standard dansylamino acids (0.5 μL), and a mixture of dansylmethionine sulfoxide and dansylmethionine sulfone (0.5 μL) was spotted onto polyamide plates (Brinkmann Instrument Co.) and chromatographed in solvents I, II, and III of Weiner et al. (1972). Unknown dansylamino acids were located from the positions of standard amino acids. Spots were cut out, suspended in 7 mL of aquasol, and counted in a scintillation counter. The results were corrected for recovery on the basis of [^{14}C]dansylalanine content.

Table I: Peptides and Amino-Terminal End Groups Used for Determination of Rates of Proteolysis at Individual Peptide Bonds

cleavage site[a]	peptides	amino terminus
Lys-13	1–13	Glu
Lys-30	1–30, 14–30	Glu
Arg-37	1–37, 14–37, 31–37	Ser
Arg-74		Lys
Lys-75	38-7Y and 7X-148[b]	Met
Lys-77		Asp
Arg-86	87–90, 87–106,[c] 87–126,[c] 87–148[c]	Glu
Arg-90	91–106, 91–126,[c] 91–148[c]	Val
Arg-106	107–126, 107–148	His
Arg-126	127–148	Glu

[a] Bonds at Lys-20 and -94, located between two Asp residues, are not cleaved by trypsin (Watterson et al., 1980). [b] Peptide 38-7Y[4] was used to measure hydrolysis rates in the presence of Mg^{2+} or/and EGTA, and peptide 7X-148[4] was used in Ca^{2+} digests. [c] Not detectable.

Assay of Trypsin. Trypsin activity was measured at 30 °C in mock proteolysis solutions containing 0.25 mM benzylarginine ethyl ester hydrochloride, 5 mg/mL bovine serum albumin, 0.05 M NH_4HCO_3, and 0.05 M NaCl, with 2 mM Ca^{2+}, 2 mM EGTA, 0.1 mM EGTA, or 0.1 mM EGTA and 1 mM Mg^{2+}. The reaction was monitored at 253 nm (Sarath et al., 1989).

Determination of Ca^{2+} Concentration. Total Ca^{2+} concentrations were determined by atomic absorption spectrophotometry with a Perkin-Elmer Model 5000 spectrometer. Free Ca^{2+} concentrations were calculated with the program of Fabiato and Fabiato (1979) using an average K_{diss} value of 5×10^{-6} M for the four Ca^{2+} sites of calmodulin.

Determination of Rates of Proteolysis. Rates were calculated from first-order rate plots. All initial rates are corrected for the minimal 1.3-fold enhancement of the activity of trypsin by concentrations of Ca^{2+} above 10^{-4} M. In the presence of calmodulin, an additional Mg^{2+} or Ca^{2+} 2-fold stimulation of the trypsin hydrolysis of benzoylarginine methyl ester, believed to be the result of competitive inhibition by additional calmodulin-susceptible bonds in the presence of EGTA, was not taken into account in these calculations.

Results

The effects of Ca^{2+} and Mg^{2+} on the conformation of calmodulin were examined by measuring their effects on the susceptibilities of individual trypsin-sensitive bonds to proteolysis. Cleavage rates at individual bonds were calculated from the sums of the peptide fragments produced by hydrolysis at each bond. The peptide fragments whose concentrations were used for measurement of cleavage rates are listed in Table I. Cleavage rates in the amino-terminal half of calmodulin are based on all possible product peptides on the amino side of the bond: cleavage at Lys-13 was based on the quantity of peptide 1–13; that at Lys-30 on the sum of peptides 1–30 and 14–30; and that at Arg-37 on the sum of fragments 1–37, 14–37, and 31–37. Cleavage rates in the carboxyl-terminal half of calmodulin are based on the amounts of the peptides produced on the carboxyl side of each bond: cleavage at Arg-126 was based on the quantity of peptide 127–148, and cleavage at Arg-106 was based on the sum of peptides 107–126

Table II: Rates of Trypsin Digestion in the Presence and Absence of Ca^{2+}

cleavage sites	rate of hydrolysis[a] (nmol mL^{-1} min^{-1})	
	Ca^{2+}	EGTA[b]
Lys-13	1.6	<1.3[c]
Lys-30	1.2	<4.8[c]
Arg-37	3.2	25
Arg-74, Lys-75, Lys-77	8.7	7.1
Arg-86	0	<6.3[c]
Arg-90	≤0.2	>160[d]
Arg-106	≤0.7	>300[d]
Arg-126	<0.007[c]	<7.3[c]

[a] Values are initial rates determined, as described under Materials and Methods. [b] Rates of digestion were the same in EGTA and EDTA. [c] A lag phase in the cleavage at these positions prevented measurement of true initial rates. [d] Rates are minimum estimates because hydrolysis at these bonds is almost complete before the first measurement at 2 min.

and 107–148. Digestion at Arg-90 was based solely on fragment 91–106, and cleavage at Arg-86 was based on the release of fragment 87–90. Other potential cleavage products, 91–126, 91–148, 87–106, 87–126, and 87–148, were not detected.

Only the sum of the rates of hydrolysis at the three bonds in the central region of calmodulin, Arg-74, Lys-75, and Lys-77, could be measured by peptide analysis because the analytical systems did not resolve the products of individual digestions at these bonds. In the presence of 2 mM Ca^{2+} to ensure saturation of all four sites, the rates are based on the quantity of peptides 7X-148,[4] a procedure that provides a good measure of the hydrolysis rate because almost no cleavage occurred in the C-domain under these conditions. In the presence of EGTA or Mg^{2+}, fragments 7X-148 could not be used because of rapid cleavages in the C-domain. Cleavage rates in the presence of EGTA, based on the rate of appearance of peptide 38-7Y, do not include potential product peptides 1-7Y, 14-7Y, and 31-7Y, and provide a minimum estimate of the overall hydrolysis rate in the region. The yields of peptides 14-7Y, 31-7Y, and 1-7Y were not determined because trypsin digestion never produced detectable quantities of the first two peptides and peptides 1-7Y were not well resolved from fragment 1–106 and CaM. To obtain a more accurate measurement of cleavage rates at Arg-74, Lys-75, and Lys-77, an independent method, based on measurement of the amino termini produced by cleavage at these bonds, was used. This analysis is possible because cleavages at Arg-74, Lys-75, and Lys-77 are the only ones yielding amino-terminal lysine, methionine, and aspartate, respectively (Table I).

Proteolysis of Calmodulin in the Presence and Absence of Ca^{2+}. In the presence of EGTA, the C-domain of calmodulin is readily digested by trypsin (Figure 1A). The rates of cleavage at Arg-106 and -90 (>300 and >160 nmol min^{-1} mL^{-1}) are too fast to be measured in these experiments (Table II). The rates of cleavage at Arg-86 and Arg-126 reproducibly exhibited lag phases which suggest that these bonds are not available to trypsin in the native structure. Postlag rates,[5] determined after the process reached linearity (14 nmol min^{-1} mL^{-1} at Arg-86 and 13 nmol min^{-1} mL^{-1} at Arg-126), reflect proteolysis of fragments resulting from prior cleavages at Arg-106 and -90. Addition of Ca^{2+} reduced the rates of hydrolysis of the trypsin-susceptible bonds in the C-domain of calmodulin by very large factors (Figure 1A and Table II).

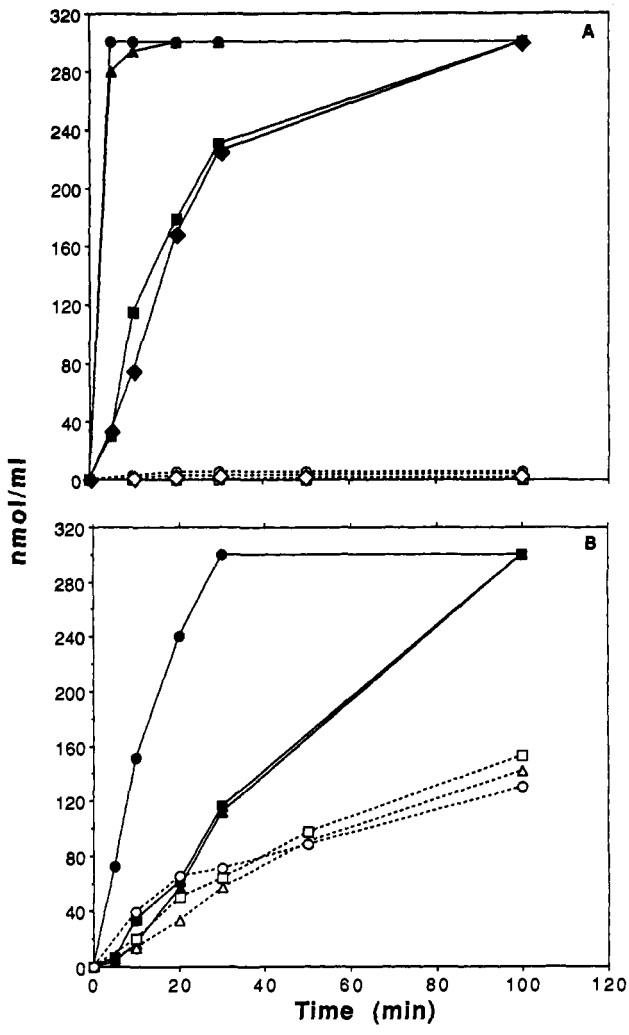

FIGURE 1: Digestion of individual peptide bonds in the presence and absence of Ca^{2+}. Calmodulin was incubated with trypsin (20 μg/mL) in the presence of either 2 mM EGTA (closed symbols) or 2 mM $CaCl_2$ (open symbols) at 30 °C as described under Materials and Methods. At the indicated times, aliquots were removed, and trypsin digestion was stopped with soybean trypsin inhibitor. The concentration of peptides used to follow proteolysis at the individual bonds (Table I) was quantitated by HPLC. (A) C-Domain of calmodulin: Arg-86 (■, □); Arg-90 (▲, △); Arg-106 (●, ○); Arg-126 (♦, ◇). (B) N-Domain of calmodulin: Lys-13 (■, □); Lys-30 (▲, △); Arg-37 (●, ○).

The hydrolysis of bonds in the N-domain of calmodulin, although rapid in EGTA, was significantly slower than was hydrolysis in the C-domain (Figure 1B and Table II). Arg-37, in the F-helix of Ca^{2+}-binding site I, a counterpart of Arg-106 in the F-helix of site III, was the first bond to be cleaved in the N-domain. Lags in the progress curves for cleavage at Lys-13 and Lys-30 indicated that cleavage of these bonds occurred more rapidly after proteolytic fragmentation of native calmodulin. Postlag rates were 5.3 and 6.1 nmol min^{-1} mL^{-1} for Lys-13 and Lys-30, respectively. Like the bond at Arg-106, the bond at Arg-37 was protected by Ca^{2+} (Figure 1B). The cleavage rate at Lys-30 was decreased in the presence of Ca^{2+}, but unlike peptide bonds at Arg-86, Arg-90, and Arg-126, the lag phases characteristic of digestion at Lys-13 and Lys-30 in EGTA were no longer detectable when Ca^{2+} was present (Figure 1B).

In the presence of EGTA, the central helical region of calmodulin was less susceptible to trypsin than were the peptide bonds at Arg-106 or Arg-37 (Table II). The total rate of hydrolysis at Arg-74, Lys-75, and Lys-77 was 7.1 nmol min^{-1}

[4] 7X is used to designate Lys-75, Met-76, and Asp-78. 7Y is used to designate Arg-74, Lys-75, or Lys-77.

[5] The postlag rates are the maximum rates determined between 5- and 10-min digestion.

Table III: Hydrolysis of Individual Bonds in the Central Helical Region of Calmodulin

conditions	rate of hydrolysis[a] (nmol mL^{-1} min^{-1})		
	Arg-74	Lys-75	Lys-77
Ca^{2+} (2 mM)	2.5 ± 0	3.0 ± 1.1	3.5 ± 0.7
EGTA (2 mM)	3.1 ± 1.1	4.2 ± 0.4	2.8 ± 0.4
EGTA (0.1 mM)	3.6 ± 0.6	4.3 ± 0.6	2.4 ± 0.5
Mg^{2+} (1 mM)	2.4 ± 0.7	3.1 ± 0.3	2.7 ± 0.3

[a] Hydrolysis rates (corrected for a 1.3-fold stimulation of trypsin activity by Ca^{2+}) were measured by quantitative end-group analysis as described under Materials and Methods.

Table IV: Rates of Trypsin Digestion in the Presence and Absence of Mg^{2+}

cleavage sites	rate of hydrolysis[a] (nmol mL^{-1} min^{-1})	
	Mg^{2+}	EGTA
Lys-13[b]	<10.4	<2.6
Lys-30[b]	<4.4	<3.9
Arg-37	15	28
Arg-74 + Lys-75 + Lys-77	≥5.6	≥16[c]
Arg-86[b]	<4.1	nd
Arg-90[b]	<99	<110
Arg-106[d]	≥230	≥260
Arg-126[b]	<9.5	<5.4

[a] Values are initial rates determined, as described under Materials and Methods, during the first 2 min of reaction. [EGTA] was 0.1 mM and [Mg^{2+}] 1 mM. [b] The lag phase prevented measurements of true initial rates. nd, not detectable. [c] This rate is greater than the rate in 2 mM EGTA (Table II). The difference is probably due to the failure of 0.1 mM EGTA to chelate all endogenous Ca^{2+}. At low concentrations, Ca^{2+} stimulates hydrolysis (see Figure 2). [d] Rates are minimum estimates because hydrolyses at these bonds are complete before the first measurement at 2 min.

mL^{-1}. This rate, based on the yield of peptide 38-7Y, is a low estimate. In the absence of Ca^{2+}, the sum of cleavage rates at Arg-74, Lys-75, and Lys-77 determined by end group quantitation was 10.1 nmol min^{-1} mL^{-1} (Table III). At 2 mM Ca^{2+}, the cumulative rate of cleavage at the three bonds (9 nmol mL^{-1} min^{-1}) was consistently smaller than in the presence of EGTA (Table III).

Limited Proteolysis of Calmodulin in the Presence and Absence of Mg^{2+}. The effects of Mg^{2+} binding on the proteolytic sensitivity of calmodulin were analyzed because Mg^{2+} has significant effects on the Ca^{2+}-binding properties of the protein. At high concentrations (>1 mM), Mg^{2+} competes with Ca^{2+} for binding sites but causes a conformational change different from that produced by Ca^{2+} [as reviewed by Klee (1988)]. At physiological concentrations (≤1 mM), Mg^{2+} increases the affinity of some of the calmodulin sites for Ca^{2+} (Iida & Potter, 1986).[6]

Mg^{2+} exerted only a small effect on the proteolysis of the C-domain of calmodulin. The rate of proteolysis at Arg-106 was reduced but much less than it was by Ca^{2+} (Tables II and IV). Cleavages at Arg-86, Arg-90, and Arg-126 exhibited lags both in the presence and in the absence of Mg^{2+}. The postlag rates at Arg-86 (20 nmol min^{-1} mL^{-1}) and Arg-90 (127 nmol min^{-1} mL^{-1}) were not significantly affected by Mg^{2+}, but the postlag rate at Arg-126 was increased from 9.5 to 19 nmol min^{-1} mL^{-1} by Mg^{2+}. In the N-domain, digestion at Arg-37 was reduced by Mg^{2+} more than the cleavage rate at Arg-106. Lags were observed at Lys-13 and -30 in both the presence and absence of Mg^{2+}. The postlag rate at Lys-30 (~8 nmol min^{-1} mL^{-1}) was not affected by Mg^{2+}, but as in the case of Arg-126, the postlag rate at Lys-13 was increased

[6] D. L. Newton, Y. Haiech, and C. B. Klee, unpublished results.

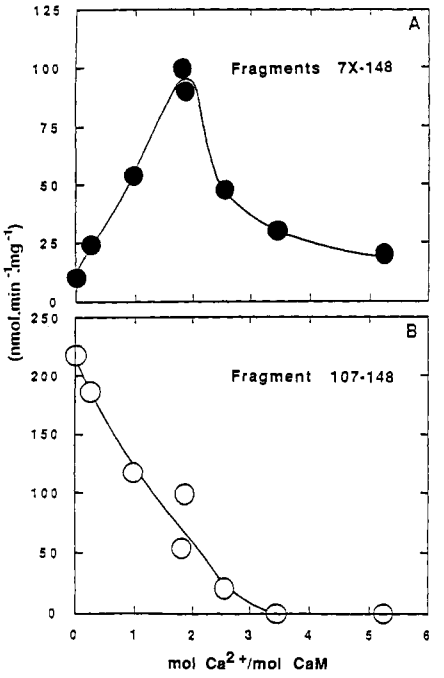

FIGURE 2: Digestion of calmodulin at varying Ca^{2+} concentrations. Calmodulin was incubated with trypsin in the presence of varying Ca^{2+} concentrations. Free Ca^{2+} concentrations, determined as described under Materials and Methods, were 0.014, 0.06, 0.16, 0.17, 0.35, 1.2, and 373 μM. With the exception of the highest Ca^{2+} addition, the Ca^{2+} effect on the trypsin activity was negligible. At 2 min, aliquots were removed, digestion was stopped, and digests were analyzed by HPLC. (A) Rates of hydrolysis at Arg-74, Lys-75, and Lys-77 (peptides 7X-148); (B) rates of hydrolysis at Arg-106 (peptide 107–148).

from 18 to 43 nmol min^{-1} mL^{-1} in the presence of Mg^{2+}.

Total hydrolysis at Arg-74, Lys-75, and Lys-77 dropped from 16 nmol min^{-1} mL^{-1} in the absence of Mg^{2+} to 5.6 nmol min^{-1} mL^{-1} when Mg^{2+} was present, and the individual rates of hydrolysis at Arg-74 and Lys-75, measured by amino-terminal analysis, were both reduced by Mg^{2+} (Table III).

Concentration Dependence of the Protective Effect of Ca^{2+} on the Susceptibility of Calmodulin to Trypsin. Since calmodulin contains four specific Ca^{2+}-binding sites and is believed to undergo specific structural changes upon Ca^{2+} binding to individual sites, the susceptibility of the trypsin-sensitive bonds in the central helix and the C-domain of calmodulin was examined at various degrees of Ca^{2+} occupancy. At 10 nmol min^{-1} mL^{-1}, the total rate of hydrolysis at Arg-74, Lys-75, and Lys-77 was lowest in the absence of Ca^{2+} (2 mM EGTA) (Figure 2A). The hydrolysis rate rose to 47 nmol min^{-1} mL^{-1} when Ca^{2+} was present at 1 equiv/mol of calmodulin (300 μM). At 2 Ca^{2+} equiv/mol, the rate reached 100 nmol min^{-1} mL^{-1}, a 10-fold increase over the basal level. As Ca^{2+} occupancy was increased further, the hydrolysis rate in the central helix decreased to a final level of 20 nmol min^{-1} mL^{-1} at saturating Ca^{2+} concentrations. The pattern of proteolysis also shifted with increasing Ca^{2+}. The cleavages at Lys-75 and -77 were accelerated whereas cleavage at Arg-74 was reduced (Table V). Mg^{2+} protected against hydrolysis at Arg-74 at all Ca^{2+} levels and accelerated hydrolysis at both Lys-75 and Lys-77 at high Ca^{2+} concentration only.

In contrast to the bonds in the central helix, the bond at Arg-106 was most susceptible to proteolysis in the absence of Ca^{2+}. As Ca^{2+} occupancy increased, the rate of hydrolysis at this bond was reduced (Figure 2B). The most significant reduction in susceptibility occurred between 0 and 2 equiv of Ca^{2+}/mol of calmodulin. Mg^{2+} (1 mM) exerted a small protective effect at all levels of Ca^{2+} occupancy (data not

Table V: Effect of Mg^{2+} on the Hydrolysis of Individual Bonds in the Central Helix of Calmodulin with Varying Ca^{2+} Concentrations[a]

	hydrolysis rate[b] (nmol min^{-1} mL^{-1})					
	−1 mM Mg^{2+}			+1 mM Mg		
mol of Ca^{2+}/mol of CaM	Arg-74	Lys-75	Lys-77	Arg-74	Lys-75	Lys-77
1.0	43	19	2.5	21	19	2
1.8	83	30	7	32	32	5
2.5	30	26	3	4	47	12

[a] Cleavage rates are rates of appearance of peptides 75–, 76–, and 78–148 determined after 2-min digestion by quantitative HPLC monitored at 280 nm. [b] Values are not corrected for the effects of metal ions on trypsin activity. When Ca^{2+} is present, Mg^{2+} has no additional effect on trypsin. Therefore, rates in the presence and absence of Mg^{2+} are directly comparable.

shown). After 2-min incubation with trypsin, calmodulin was cleaved either in the C-domain, at Arg-106 (and Arg-90 in the presence of EGTA), or in the central helix, at Arg-74 and Lys-75 and -77, but never in the C-domain and in the central helix at the same time. Concomitant cleavages in the C-domain and the central helix would produce 280-nm-absorbing peptides, 7X-106, which were never detected in our experiments. Cleavage at Arg-74 was always accompanied by cleavage at Arg-37, yielding equimolar amounts of peptides 1–37 and 38-7X.

Discussion

Our results agree with and extend the conclusions concerning the structure of calmodulin in the Ca^{2+}-saturated and Ca^{2+}-free forms reached on the basis of NMR (Evans et al., 1988) and fluorescence anisotropy studies (Lambooy et al., 1982). In the absence of Ca^{2+}, the C-domain and, to a lesser extent, the N-domain of calmodulin are apparently flexible and therefore rapidly proteolyzed. The peptide bond at Arg-106, in the F-helix of domain III, is rapidly cleaved by trypsin in the absence of Ca^{2+} as reported earlier (Drabikowski et al., 1977; Kawasaki et al., 1986). Cleavage of the three remaining bonds of the C-domain, at Arg-86, Arg-90, and Arg-126, occurs with a lag suggesting that these bonds are not available to trypsin in the native molecule. The postlag cleavage rates reflect proteolysis of peptides resulting from the cleavage at Arg-106. In the presence of excess Ca^{2+}, each of bonds in the C-domain becomes almost completely resistant to proteolysis. Thus, Ca^{2+} induces a major conformational change in the C-domain of calmodulin. In the N-domain, Arg-37 is also susceptible to proteolysis in the absence of Ca^{2+}, but its rate of cleavage is significantly lower than that of Arg-106 as reported by Kawasaki et al. (1986). Since the amino acid sequence around Arg-37 is similar to that around Arg-106, we suggest that the different rates show that the F-helix of domain I is more stable than that of domain III. The observed lags in cleavages at Lys-13 and Lys-30 suggest that these cleavages require prior cleavage of calmodulin, probably at Arg-37. The rate of cleavage at Arg-37 is significantly decreased in Ca^{2+}-saturated calmodulin, but the protection is less marked than in the C-domain. The high Ca^{2+} off-rates from sites I and II (Drakenberg et al., 1983; Ikura et al., 1984; Martin et al., 1985; Suko et al., 1986) may provide windows for the irreversible action of trypsin. Although the sequences around Arg-86 and Lys-13 are similar, cleavage at Arg-86 is prevented by Ca^{2+} whereas proteolysis at Lys-13 is facilitated, suggesting that the conformational changes induced by Ca^{2+} in the isolated N- and C-domains are quite different.

By molecular modeling based upon troponin C, Strynacka and James (1988) predicted that Arg-106 and Arg-37 of calmodulin are involved in salt bridges with Asp-118 and Glu-45, respectively, in both the Ca^{2+}-free and Ca^{2+}-saturated calmodulin. Their predictions imply that the helix contents of the two forms of calmodulin are the same. The results of our experiments are not easily compatible with this proposal but are consistent with the small change (5–8%) in helix content reported earlier (Klee & Vanaman, 1982; Hennessey et al., 1987) and suggest that the E- and F-helices of domain III are the ones most affected by Ca^{2+}. The Ca^{2+}-induced chemical shift of Asp-118 reported by Ikura et al. (1985) also suggests that the salt bridge with Arg-106 is missing in the Ca^{2+}-free calmodulin. It has been proposed, on the basis of Fourier-transformed infrared spectroscopic studies, that Ca^{2+} does not induce an increase in α-helix content but produces a distortion of the helices (Trewhella et al., 1989). If this were so, the distorted helices would have to be less susceptible to proteolysis than regular α-helices.

Saturating amounts of Ca^{2+} have a small protective effect on the susceptibility of the central helix to proteolysis. Earlier experiments in which proteolysis produced fragments 1–77 and 78–148 in the presence of Ca^{2+} and fragments 1–106 and 107–148 in its absence were conducted under different conditions (Walsh et al., 1977). These patterns were not the result of preferential cleavage at 74–77 in the presence of Ca^{2+}, as previously suggested, but were probably due to the fact that at the very low levels of trypsin used in the EGTA digests the slower cleavage in the central helix was not observed.

Mg^{2+} has only small effects on the rates of proteolysis of the N- and C-domains of calmodulin. Its protective effects are more pronounced in the N-domain than in the C-domain, in agreement with the preferential binding of Mg^{2+} to sites I and II (Tsai et al., 1987).[6] The largest effect of Mg^{2+} is its protection of the central helix. The stabilization of the central helix by Ca^{2+} and Mg^{2+}, as opposed the large difference in responses of the rest of the molecule to the two metals, suggests the existence of nonselective Ca^{2+}/Mg^{2+} sites with a stabilizing effect on this part of the molecule.

The most striking and novel effect of Ca^{2+} observed is a 10-20-fold increase in susceptibility to trypsin of the peptide bonds in the central helix upon *partial* occupancy of the Ca^{2+} sites. The high proteolysis rate cannot be explained by a mixture of Ca^{2+}-free and Ca^{2+}-saturated forms of calmodulin since these bonds are attacked only slowly by trypsin in both extreme forms. The lack of detectable amounts of fragments 75–106, 76–106, and 78–106 and the good correlation between the increased rate of proteolysis at Arg-74, Lys-75, and Lys-77 and decreased rate at Arg-106 indicate that the structural rearrangement preventing cleavage at Arg-106 facilitates proteolysis at Arg-74. The almost complete protection at Arg-106, -86, and -90 and maximum sensitization of the bonds in the central helix are observed at 2 Ca^{2+}/mol of calmodulin, suggesting that the cooperative binding of 2 mol of Ca^{2+} to the high-affinity sites of calmodulin (previously identified as sites III and IV) generates a kinetically significant calmodulin conformer with a unique conformation of the central helix that exposes its basic residues to trypsin. The existence of multiple conformational substates of calmodulin in solution has been proposed to explain the discrepancy between the long dumbell shape molecule revealed by the crystallographic data and the

more compact molecule predicted on the basis of small-angle X-ray scattering spectra (Heidorn & Trewhella, 1988). According to our data, Ca^{2+}-free calmodulin and Ca^{2+}-saturated calmodulin exist as elongated molecules with a central helix resistant to proteolysis whereas the partially loaded calmodulin may be bent or flexible and therefore less asymmetric. The multiple substates postulated by Heidorn and Trewhella (1988) may reflect the presence of intermediates partially loaded with Ca^{2+}. These intermediates are selectively tracked in our experiments because of the irreversibility of the trypsin assay. The flexibility of the calmodulin molecule in the central helix may play an important role in allowing calmodulin to fit the many different calmodulin-binding domains of its targets.

ACKNOWLEDGMENTS

We thank Dr. Paul Stemmer for the determinations of total Ca^{2+} concentrations, Joseph Gomez for the drawing of the figures, and May Liu for expert editorial assistance.

Registry No. Ca^{2+}, 7440-70-2; Mg^{2+}, 7439-95-4; trypsin, 9002-07-7.

REFERENCES

Aulabaugh, A., Niemczura, W. P., & Gibbons, W. A. (1984) *Biochem. Biophys. Res. Commun. 118*, 225–232.

Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) *Nature 315*, 37–40.

Babu, Y. S., Bugg, C. E., & Cook, W. J. (1988) *J. Mol. Biol. 204*, 191–204.

Dalgarno, D. C., Klevit, R. E., Levine, B. A., Williams, R. J. P., Dobrowolski, Z., & Drabikowski, W. (1984) *Eur. J. Biochem. 138*, 281–289.

Drabikowski, W., Kuznicki, J., & Grabarek, Z. (1977) *Biochim. Biophys. Acta 485*, 124–133.

Drakenberg, T., Forsen, S., & Lilja, H. (1983) *J. Magn. Reson. 53*, 412–422.

Evans, J. S., Levine, B. A., Williams, R. J. P., & Wormald, M. R. (1988) in *Calmodulin, Molecular Aspects of Cellular Regulation* (Cohen, P., & Klee, C. B., Eds.) Vol. 5, pp 57–82, Elsevier, New York.

Fabiato, A., & Fabiato, F. (1979) *J. Physiol. (Paris) 75*, 463–505.

Forsen, S., Vogel, H. J., & Drakenberg, T. (1986) *Calcium Cell Funct. 6*, 113–157.

Heidorn, D. B., & Trewhella, J. (1988) *Biochemistry 27*, 909–915.

Heidorn, D. B., Seeger, P. A., Rokop, S. E., Blumenthal, D. K., Means, A. R., Crespi, H., & Trewhella, J. (1989) *Biochemistry 28*, 6757–6764.

Hennessey, J. P., Jr., Manavalan, P., Johnson, W. C., Jr., Malencik, D. A., Anderson, S. R., Schimerlik, M. I., & Shalitin, Y. (1987) *Biopolymers 26*, 561–571.

Iida, S., & Potter, J. D. (1986) *J. Biochem. 99*, 1765–1772.

Ikura, M., Hiraoki, T., Hikichi, K., Minowa, O., Yamaguchi, H., Yasawa, M., & Yagi, K. (1984) *Biochemistry 23*, 3124–3128.

Ikura, M., Minowa, O., & Hikichi, K. (1985) *Biochemistry 24*, 4264–4269.

Ikura, M., Hasegawa, N., Aimoto, S., Yasawa, M., Yagi, K., & Hikichi, K. (1989) *Biochem. Biophys. Res. Commun. 161*, 1233–1238.

Kataoka, M., Head, J. F., Seaton, B. A., & Engelman, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6944–6948.

Kawasaki, H., Kurosu, Y., Hisataka, K., Isobe, T., & Okuyama, T. (1986) *J. Biochem. 99*, 1409–1416.

Klee, C. B. (1988) in *Calmodulin, Molecular Aspects of Cellular Regulation* (Cohen, P., & Klee, C. B., Eds.) Vol. 5, pp 35–56, Elsevier, New York.

Klee, C. B., & Vanaman, T. C. (1982) *Adv. Protein Chem. 35*, 213–323.

Klevit, R. E., Dalgarno, D. C., Levine, B. A., & Williams, R. J. P. (1984) *Eur. J. Biochem. 139*, 109–114.

Kretsinger, R. H., Rudnick, S. E., & Weissman, L. J. (1986) *J. Inorg. Biochem. 28*, 289–302.

Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W., & Cohen, P. (1981) *FEBS Lett. 130*, 141–145.

Lambooy, P. K., Steiner, R. F., & Sternberg, H. (1982) *Arch. Biochem. Biophys. 217*, 517–528.

LaPorte, D. C., Wierman, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814–3819.

Manalan, A. S., & Klee, C. B. (1987) *Biochemistry 26*, 1382–1390.

Martin, S. R., Andersson-Teleman, A., Bayley, P. M., Drakenberg, T., & Forsen, S. (1985) *Eur. J. Biochem. 151*, 543–550.

Minowa, O., & Yagi, K. (1984) *J. Biochem. 96*, 1175–1182.

Newton, D. L., & Klee, C. B. (1989) *Biochemistry 28*, 3750–3757.

Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., & Klee, C. B. (1984) *J. Biol. Chem. 259*, 4419–4426.

Newton, D. L., Krinks, M. H., Kaufman, J. B., Shiloach, J., & Klee, C. B. (1988) *Prep. Biochem. 18*, 247–259.

O'Neil, K. T., Erickson-Viitanen, S., & DeGrado, W. F. (1989) *J. Biol. Chem. 264*, 14571–14578.

Persechini, A., & Kretsinger, R. H. (1988) *J. Biol. Chem. 263*, 12175–12178.

Putkey, S. A., Ono, T., Van Berkum, M. F. A., & Means, A. R. (1988) *J. Biol. Chem. 263*, 11242–11249.

Sarath, G., de la Motte, R. S., & Wagner, F. W. (1989) in *Proteolytic Enzymes* (Beynon, R. J., & Bond, J. S., Eds.) pp 25–55, IRL Press, New York.

Seaton, B. A., Head, J. F., Engelman, D. M., & Richards, F. M. (1985) *Biochemistry 24*, 6740–6743.

Strynadka, N. C. J., & James, M. N. G. (1988) *Proteins: Struct., Funct., Genet. 3*, 1–17.

Suko, J., Wyskovsky, W., Pidlich, J., Hauptner, R., Plank, B., & Hellmann, G. (1986) *Eur. J. Biochem. 159*, 425–434.

Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078–11080.

Trewhella, J., Liddle, W. K., Heidorn, D. B., & Strynacka, N. (1989) *Biochemistry 28*, 1294–1301.

Tsai, M.-D., Drakenberg, T., Thulin, E., & Forsen, S. (1987) *Biochemistry 26*, 3635–3643.

Tsalkova, T. N., & Privalov, P. L. (1985) *J. Mol. Biol. 181*, 533–544.

Van Berkum, M. F. A., George, S. E., & Means, A. R. (1990) *J. Biol. Chem. 265*, 3750–3756.

Vogel, H. J., Lindahl, L., & Thulin, E. (1983) *FEBS Lett. 157*, 241–246.

Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977) *J. Biol. Chem. 252*, 7740–7743.

Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962–975.

Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247*, 3242–3251.

Yoshino, H., Minari, O., Matsushima, N., Ueki, T., Miyake, Y., Matsuo, T., & Izumi, Y. (1989) *J. Biol. Chem. 264*, 19706–19709.