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Structural analysis of wild-type and mutant yeast calmodulins by limited proteolysis and electrospray ionization mass spectrometry

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Abstract

Calmodulin from *Saccharomyces cerevisiae* was expressed in *Escherichia coli* and purified. The purified protein was structurally characterized using limited proteolysis followed by ESI mass spectrometry to identify the fragments. In the presence of Ca^{2+}, yeast calmodulin is sequentially cleaved at arginine 126, then lysine 115, and finally at lysine 77. The rapid cleavage at Arg-126 suggests that the fourth Ca^{2+}-binding loop does not bind Ca^{2+}. In the presence of EGTA, yeast calmodulin is more susceptible to proteolysis and is preferentially cleaved at Lys-106. In addition, mutant proteins carrying I100N, E104V or both mutations, which together confer temperature sensitivity to yeast, were characterized. The mutant proteins are more susceptible than wild-type calmodulin to proteolysis, suggesting that each mutation disrupts the structure of calmodulin. Furthermore, whereas wild-type calmodulin is cut at Lys-106 only in the presence of EGTA, this cleavage site is accessible in the mutants in the presence of Ca^{2+} as well. In these ways, the structural consequence of each mutation mimics the loss of a calcium ion in the third loop. In addition, although wild-type calmodulin binds to four proteins in a yeast crude extract in the presence of Ca^{2+}, the mutants bind only to a subset of these. Thus, the inability to adopt the stable Ca^{2+}-bound conformation in the third Ca^{2+}-binding loop alters the ability of calmodulin to interact with yeast proteins in a Ca^{2+}-dependent manner.

Keywords: calcium-binding properties; calmodulin; ESI mass spectrometry; mutant; wild type; yeast

Calmodulin is a conserved Ca^{2+}-binding protein that is recognized as the Ca^{2+}-dependent regulator of many enzymes including cyclic nucleotide phosphodiesterase, adenylate cyclase, Ca^{2+}-Mg^{2+} ATPase, calcineurin, Ca^{2+}-calmodulin-dependent protein kinase, phosphorylase kinase, and myosin light chain kinase (for review see Cohen & Klee, 1988). It is the Ca^{2+} receptor that regulates muscle contraction in smooth muscle cells and glycogen breakdown in muscle and liver cells. In addition, calmodulin is required for cellular growth and division (Davis et al., 1986; Takeda & Yamamoto, 1987; Rasmussen & Means, 1989; Rasmussen et al., 1990). Mutant calmodulins in which all three Ca^{2+}-binding sites have been inactivated can support the growth of yeast cells (Geiser et al., 1991), suggesting that calmodulin can perform its required function without binding Ca^{2+}.

The crystal structure of vertebrate Ca_4^{2+}-calmodulin reveals a dumbbell-shaped protein with two globular domains connected by a long central helix (Kinemage 1; Babu et al., 1988). Each domain contains two EF hands, which are helix-loop-helix Ca^{2+}-binding structures. Thus, vertebrate calmodulin binds a total of four calcium ions. A review of 355 EF hand structures (Kretsinger et al., 1991), 10 of them solved crystallographically (Kretsinger & Nockolds, 1973; Babu et al., 1988; Herzberg & James, 1988), has established a consensus amino acid sequence. Multidimensional NMR of Ca_4^{2+}-calmodulin indicates that the middle of the central helix (residues 77–82) is flexible in solution (Ikura et al., 1991). Neither the crystal structure nor the solution structure of apocalmodulin is known.

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Abbreviations: I100N, the Ile 100 to Asn mutation; E104V, the Glu 104 to Val mutation; CamE104V, calmodulin containing E104V; CamI100N, calmodulin containing I100N; CamINEV, calmodulin containing both E104V and I100N; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; ESI, electrospray ionization; FPLC, fast performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

As part of an investigation into the critical role of calmodulin in the yeast *Saccharomyces cerevisiae*, we are studying the structure of yeast calmodulin. Little structural information is currently available for yeast calmodulin. Several lines of evidence suggest that yeast calmodulin adopts a structure with similarities to vertebrate calmodulin, but with significant differences. First, the sequence shares 60% identity with vertebrate calmodulin (Davis et al., 1986). Second, the secondary structure of yeast calmodulin as predicted by a Chou and Fasman algorithm is similar to the secondary structure of vertebrate calmodulin (Davis & Thorner, 1987). Finally and most significantly, vertebrate calmodulin can perform all the calmodulin functions required for yeast cell growth. Yeast cells in which the endogenous calmodulin gene is replaced by a cDNA encoding vertebrate calmodulin grow well (Davis & Thorner, 1989; Ohya & Anraku, 1989; Persechini et al., 1991). Thus, the structural elements required for yeast growth are conserved in vertebrate calmodulin.

The significant structural differences between yeast and vertebrate calmodulin are reflected in the high concentration of yeast calmodulin required to activate vertebrate enzymes. The concentration of yeast calmodulin that half-maximally activates brain cyclic nucleotide phosphodiesterase is 100-fold larger than the K_{act} for activation of this enzyme by vertebrate calmodulin (Luan et al., 1987; Davis & Thorner, 1989). Even more striking, the K_{act} for activation of chicken gizzard muscle myosin light chain kinase by yeast calmodulin is 1,000-fold larger than the K_{act} for activation by vertebrate calmodulin (Luan et al., 1987).

Another striking structural difference is that yeast calmodulin binds only three calcium ions with high affinity (Luan et al., 1987; T.N. Davis, unpubl.), whereas vertebrate calmodulin binds four (Haiech et al., 1981). Although a prediction of the secondary structure of yeast calmodulin predicts four helix–loop–helix structures, only the first three loops have all the residues required to ligand a calcium ion (Davis et al., 1986).

Initial structural analyses of vertebrate calmodulin included a detailed analysis of the fragments released after partial digestion with trypsin (Walsh et al., 1977; Newton et al., 1984; Thulin et al., 1984). These studies provided substantial information about the domain structure of vertebrate calmodulin before the crystal structure was available. Furthermore, they demonstrated that apocalmodulin is significantly more susceptible to proteolysis and is completely degraded under conditions that only cleave Ca^{2+}-calmodulin into two domains.

To provide the first structural analysis of yeast calmodulin and compare it to that of vertebrate calmodulin we have subjected yeast calmodulin to partial digestion by trypsin in the presence of either Ca^{2+} or EGTA and identified the resulting fragments. A difficult aspect of such an analysis is identification of the many fragments produced during proteolysis. In the past, definitive identification has required purification of each fragment, followed by amino acid analysis and N-terminal determination (Newton et al., 1984). With the advent of ESI mass spectrometry (Fenn et al., 1989), the mass of each fragment in a mixture can be determined very accurately (here, within 1–4 Da). This degree of accuracy allows identification of the calmodulin fragments, for which the sequence is known, based on mass alone and obviates the need for purification.

A temperature-sensitive calmodulin mutant was recently isolated and characterized. The mutant protein (CamINEV) has two amino acid substitutions in the third Ca^{2+}-binding loop (Kinemage 1); isoleucine 100 is changed to an asparagine (I100N), and glutamic acid 104 is changed to a valine (E104V). Because the mutations have a dramatic effect on the function of calmodulin in vivo, we have extended the present study to determine how these mutations affect the structure and stability of yeast calmodulin.

Results

Expression of yeast calmodulin in Escherichia coli

Mutant and wild-type yeast calmodulin were produced in *E. coli*. Figure 1 is a representative nondenaturing gel of crude *E. coli* extracts. Densitometric comparison of the amount of calmodulin in the extracts with known amounts of purified calmodulin showed that approximately 5% of the protein in the extract is CamI100N, CamE104V, or CamINEV, while 10–20% is wild-type calmodulin.

Wild-type and mutant calmodulins were purified from *E. coli* extracts (see Materials and methods). The relative molecular masses for wild-type calmodulin, CamI100N, CamE104V, and CamINEV as determined by ESI mass spectrometry were 15,988.0 ± 4.3, 15,989.6 ± 2.9, 15,958.2 ± 2.6, and 15,960.8 ± 2.3, respectively. These observed values are in excellent agreement with the values calculated by assuming that the only modification is removal of the initial methionine and allowing for the substitution of S1A in recombinant calmodulin (see Materials and methods), namely 15,987.6, 15,988.6, 15,957.7, and 15,958.6, respectively (Bairoch, 1982; Creighton, 1983).

Partial proteolysis of wild-type yeast calmodulin

In the presence of Ca^{2+}, wild-type calmodulin was sequentially cleaved at three sites by trypsin (Fig. 2A). N-terminal sequence analysis of the three fragments indicated that they all had the same N-terminal sequence as the whole protein (and confirmed that the initial methionine was removed in *E. coli*). Thus, digestion proceeded from the C-terminus toward the N-terminus. To

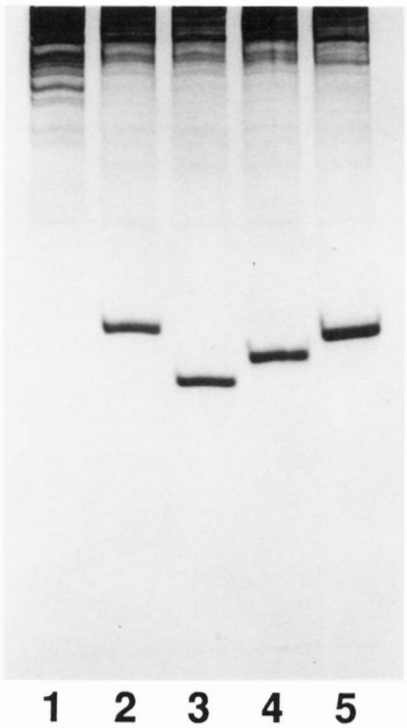

Fig. 1. Expression of wild-type and mutant forms of yeast calmodulin in *Escherichia coli*. Calmodulin was produced as described (Geiser et al., 1991) in *E. coli* strain GM1 carrying the lysis plasmid pSB6 (Geiser et al., 1991) and plasmid pSB11, pSB12, pSB13, or pJG28. Crude extracts (15 μg) were subjected to electrophoresis in a nondenaturing gel. Lane 1 contains an extract of strain GM1 containing only the lysis plasmid pSB6. Lanes 2–5 contain extracts containing CamI100N, CamE104V, CamINEV, and wild-type calmodulin, respectively.

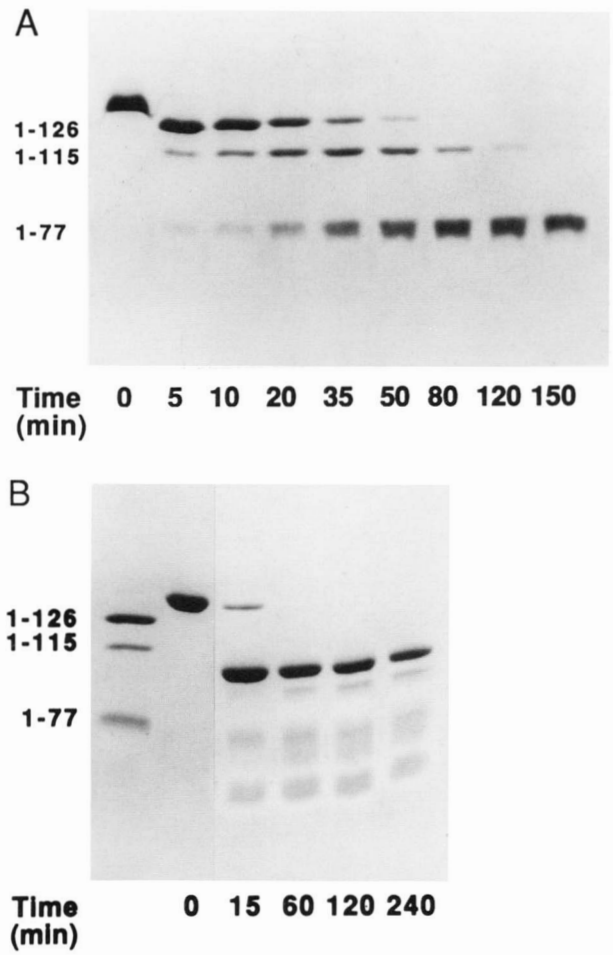

Fig. 2. Partial tryptic digest of wild-type calmodulin. **A:** Wild-type yeast calmodulin was digested in buffer containing 1 mM $CaCl_2$. The trypsin/calmodulin weight ratio was 1:62.5. At the indicated times soybean trypsin inhibitor was added to an aliquot of the digestion mixture (1.4 mg soybean trypsin inhibitor/1.0 mg trypsin). Laemmli sample buffer (2×) containing 2 mM EGTA was then added to this aliquot, and a portion was analyzed on an SDS polyacrylamide gel. The 0 time point contains undigested wild-type calmodulin. Each lane in panel A contains the products of 3 μg of calmodulin. **B:** Digestion was done in buffer containing 1 mM EGTA at a trypsin/calmodulin weight ratio of 1:500. For comparison, the first lane contains a sample of wild-type calmodulin after partial digestion with trypsin in the presence of Ca^{2+}. Each lane contains the products of 2.5 μg of calmodulin.

identify the cleavage sites, we purified the fragments using reverse-phase HPLC (see Materials and methods). The molecular weights of the largest, middle, and smallest fragments were determined by ESI mass spectrometry to be 13,899.8 ± 2.2, 12,642.0 ± 1.7, and 8,524.6 ± 0.3, respectively. This unequivocally identifies them as containing amino acid residues 1–126, 1–115, and 1–77, as the predicted molecular weight for each fragment is 13,900.1, 12,640.7, and 8,524.9, respectively. Thus, in the presence of Ca^{2+}, yeast calmodulin is sequentially cleaved at Arg-126, then at Lys-115, and finally at Lys-77. Cleavage at Arg-126 is very rapid. Even at a low ratio of trypsin to calmodulin (1:300, Fig. 3), approximately a third of the intact protein was cleaved at 126 in only 3 min. Fragment 1–126, however, is stable and persists even after 50 min at a fivefold higher trypsin/calmodulin ratio (1:62.5, Fig. 2A).

In the presence of EGTA, at a trypsin/calmodulin ratio of 1:500, wild-type calmodulin was nearly completely converted to one predominant fragment in 15 min (Fig. 2B). The fragment had the same N-terminus as the intact protein and an electrophoretic mobility between that of fragment 1–115 and fragment 1–77 (Fig. 2B). Another fragment, which was produced in sufficient quantities to analyze and was obvious on nondenaturing gels, had an N-terminus at His-107. Thus, the predominant fragment was tentatively identified as fragment 1–106. Fragments 1–126 and 1–115 were not detected on the SDS gel (Fig. 2B).

To avoid the time-consuming purification of fragments by HPLC we explored whether proteolytic fragments could be identified by ESI mass spectrometry when present in a mixture. First, the molecular weight of every

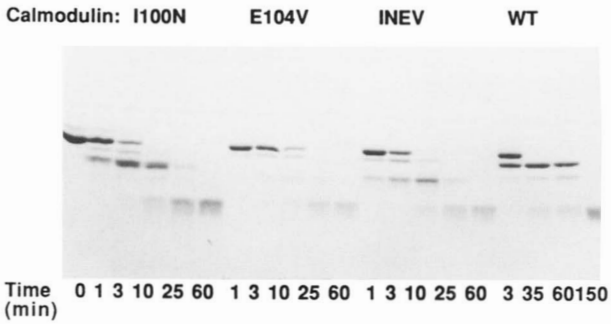

Fig. 3. Partial digests of wild-type and mutant forms of calmodulin performed in the presence of Ca^{2+}. The trypsin/calmodulin weight ratio was 1:300 except the 150-min wild-type time point, which contained a ratio of trypsin to calmodulin of 1:62.5. The experiment was conducted as described in the Materials and methods and the legend for Figure 2. Digestions of wild-type and mutant forms of calmodulin were done simultaneously. Each lane contained the products of 2.0 μg of calmodulin.

Table 1. *Fragments produced by partial proteolysis of mutant and wild-type (wt) yeast calmodulin with trypsin*[a]

	Calcium[b]					EGTA[c]			
Fragment	wt[d]	wt[e]	I100N	E104V	INEV	wt[d]	I100N	E104V	INEV
1–126	+	+	+	+	+				
1–115	+	+	+						
1–106			+	+	+	+	+	+	+
1–90						+	+		+
1–77		+	+	+	+		+		
75–146						+		+	+
107–146			+	+	+	+	+	+	+
38–74		+				+	+	+	+
1–37		+				+	+	+	
75–106						+	+		

[a] Except as noted, the trypsin/calmodulin ratios used and the time points analyzed were the same as described in the legend to Figure 6. The samples were prepared as described in the Materials and methods, and the fragments were identified by ESI mass spectrometry.
[b] Digest done in 1 mM $CaCl_2$.
[c] Digest done in 1 mM EGTA.
[d] Thirty minutes at a trypsin/calmodulin ratio of 1:500.
[e] Twenty minutes at a trypsin/calmodulin ratio of 1:62.5.

possible tryptic fragment was calculated. Yeast calmodulin has 11 arginine and lysine residues (one of which is the C-terminal residue), and thus partial proteolysis with trypsin could produce 65 different fragments. Only two of these, fragments 22–90 and 38–106, have molecular weights too close to be reliably distinguished by mass spectrometry in the present experiment (7,633 and 7,629). Neither of these fragments was detected in any of the proteolytic digests performed.

The ESI mass spectra of calmodulin digested in the presence of Ca^{2+} or EGTA (Fig. 4) were complex but were readily interpretable because the masses of the possible fragments were known. All the major responses and many of the minor responses were assigned to one of the predicted tryptic fragments (Fig. 4). The minor responses labeled g and k in Figure 4 correspond to relative molecular masses differing by at least 200 Da from any of the predicted fragments. They were observed in all digested samples and may represent contaminants.

All three calmodulin fragments, 1–126, 1–115, and 1–77, shown above to be present in a partial digest of Ca_3^{2+}-calmodulin were readily detected by ESI mass spectrometry of a mixture of fragments produced under identical conditions (trypsin/calmodulin ratio of 1:62.5 for 20 min). The responses corresponding to these fragments are labeled a, b, and e, respectively, in Figure 4. In addition, the N-terminal fragments 1–37 and 38–74 could be detected in some samples (Fig. 4; Table 1). ESI mass spectrometry of the partial digest of calmodulin in the presence of EGTA confirmed the presence of fragments 1–106 and 107–146. Consistent with the results from the SDS gels, fragments 1–126 and 1–115 were not observed (Fig. 4; Table 1). In addition, ESI mass spectrometry revealed fragments 1–90, 75–146, and 75–106 as well as the N-terminal fragments 1–37 and 38–74 (Fig. 4; Table 1). Thus, the use of ESI mass spectrometry confirmed the presence of the fragments predicted from electrophoretic mobility and allowed detection of fragments not readily observed on the SDS polyacrylamide gel.

As a final control, a mixing experiment was performed to evaluate whether the responses from certain fragments might be suppressed or otherwise obscured by a strong signal from other constituents (Fig. 5). The digest of Ca_3^{2+}-calmodulin was combined with the digest of apocalmodulin in a 1:1 mixture and then analyzed by ESI mass spectrometry (Fig. 5). Several responses from different fragments overlapped at the resolution used (for example, c^{12+} and b^{13+}, middle panel, Fig. 5). However, because each fragment yields several responses due to multiple charging, overlap of a single response did not prevent the detection of the fragment. Every fragment detected in either digest was also detected when the digests were combined. This result further demonstrates that the small fragments that gave minor responses and that were poorly resolved on the gel could be detected in the presence of the fragments that gave strong responses such as 1–126 (a), 1–115 (b), and 107–146 (h).

In conclusion, upon binding Ca^{2+}, wild-type yeast calmodulin undergoes a substantial conformational change that exposes different residues to tryptic cleavage. In the presence of EGTA, at a trypsin/calmodulin ratio of 1:500, the predominant cleavage site is at Lys-106, but cleavages at residues 37, 74, and 90 were also observed. Under identical conditions, Ca_3^{2+}-calmodulin was predominantly cleaved at Arg-126 with a low level of cleav-

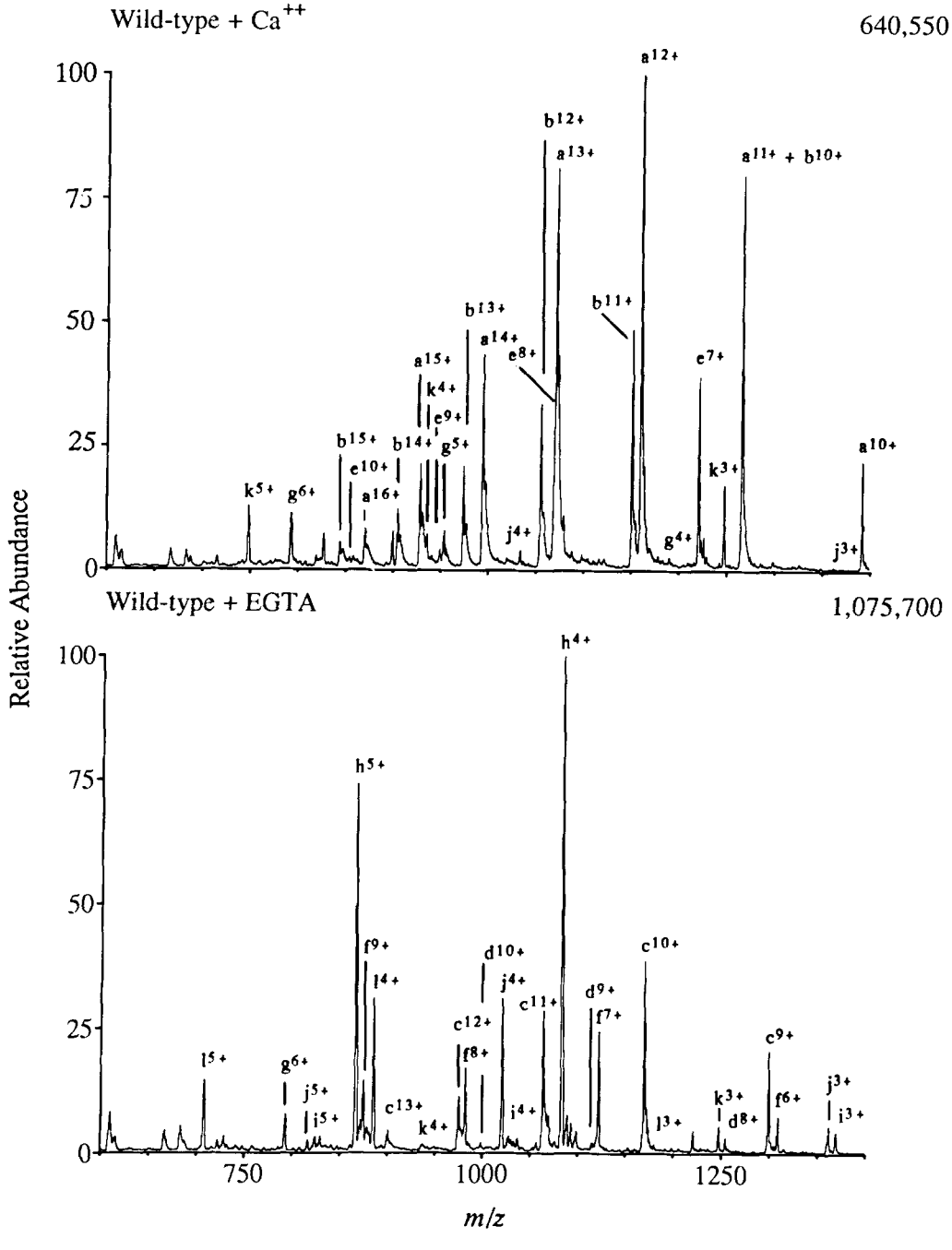

Fig. 4. ESI mass spectra of partial tryptic digests of wild-type yeast calmodulin. The upper panel is a spectrum of wild-type calmodulin digested for 20 min at a trypsin/calmodulin ratio of 1:62.5 in the presence of Ca^{2+}. The lower panel is a spectrum of wild-type calmodulin digested for 30 min at a ratio of 1:500 in EGTA. The charge state of the observed ion is indicated by the superscript (e.g., x^{n+}). The observed responses were assigned as follows: a, 1-126, M_r 13,916; b, 1-115, M_r 12,657; c, 1-106, M_r 11,692; d, 1-90, M_r 10,033; e, 1-77 M_r 8,541; f, 75-146, M_r 7,851; g, unassigned, M_r 4,758; h, 107-146, M_r 4,331; i, 38-74, M_r 4,109; j, 1-37, M_r 4,081; k, unassigned, M_r 3,740; l, 75-106, M_r 3,538.

age occurring at Lys-115 (Table 1 and data not shown). Thus, in the presence of EGTA, Lys-106 is more susceptible to proteolytic cleavage than Arg-126 or Lys-115, and the amino terminus is more susceptible to proteolytic cleavage. The results are summarized in Figure 6 and Table 1.

Partial proteolysis of the mutant calmodulins

A temperature-sensitive calmodulin mutant was isolated as described in the Materials and methods. The calmodulin gene isolated from the mutant yeast strain encodes a protein (CamINEV) with two amino acid substitutions

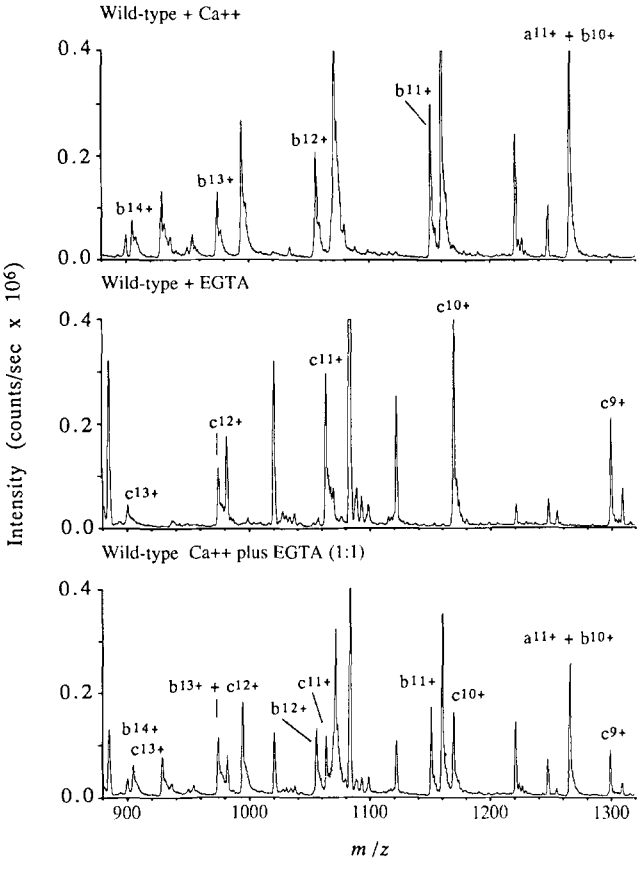

Fig. 5. ESI mass spectra of a mixture of proteolytic fragments of wild-type yeast calmodulin. An enlargement of the region that covers the distribution of responses assigned from the tryptic fragments 1-115 (b^{n+}) and 1-106 (c^{n+}) in the ESI mass spectra shown in Figure 4. Spectrum of wild-type calmodulin digested by trypsin in the presence of Ca^{2+} (upper panel), in the presence of EGTA (middle panel), or a combination of equal volumes of these digests (lower panel) is shown. Note that at the resolution used, b^{13+} (*m/z* 974.6) and c^{12+} (*m/z* 975.3) are not resolved in the combined experiment (lower panel).

in the third Ca^{2+}-binding loop; isoleucine 100 is changed to an asparagine (I100N), and glutamic acid 104 is changed to a valine (E104V). The presence of each mutation made calmodulin more susceptible to proteolysis (Fig. 3). In the presence of $CaCl_2$, at a trypsin/calmodulin ratio of 1:300, purified calmodulins containing either E104V, I100N, or both mutations were digested to the size of the half molecule in 60 min. In contrast, under identical conditions, all three wild-type fragments (1-126, 1-115, and 1-77) persisted after 60 min. Wild-type calmodulin was completely digested to the size of the half molecule only after 150 min at a fivefold higher ratio of trypsin to calmodulin than that used in the mutant digests (lane 150, Fig. 3). The mutant calmodulins were converted to half molecules 10-15 times faster than wild-type calmodulin. In addition, digestion of the mutant forms of calmodulin appeared to produce different fragments than digestion of wild type. Thus, the presence of each mutation alters the flexibility of calmodulin such that different residues are accessible to tryptic cleavage.

To define more precisely how the individual mutations alter calmodulin structure we used ESI mass spectrometry to identify the fragments produced by cleavage of the mutant forms in the presence of Ca^{2+}. Like wild-type calmodulin, CamI100N was cleaved at the C-terminal side of residues 126, 115, and 77. In addition, two other fragments were present in the crude mixture, fragment 1-106 and fragment 107-146 (Table 1). The results were identical for CamE104V and CamINEV except that fragment 1-115 was not observed in the proteolytic mixtures for these mutant forms of calmodulin (Table 1). Thus, unlike wild-type Ca_3^{2+}-calmodulin, the mutant forms of calmodulin are cleaved at the C-terminal side of Lys-106. Wild-type calmodulin is cleaved at Lys-106 only in the presence of EGTA. The mutant forms are not cleaved se-

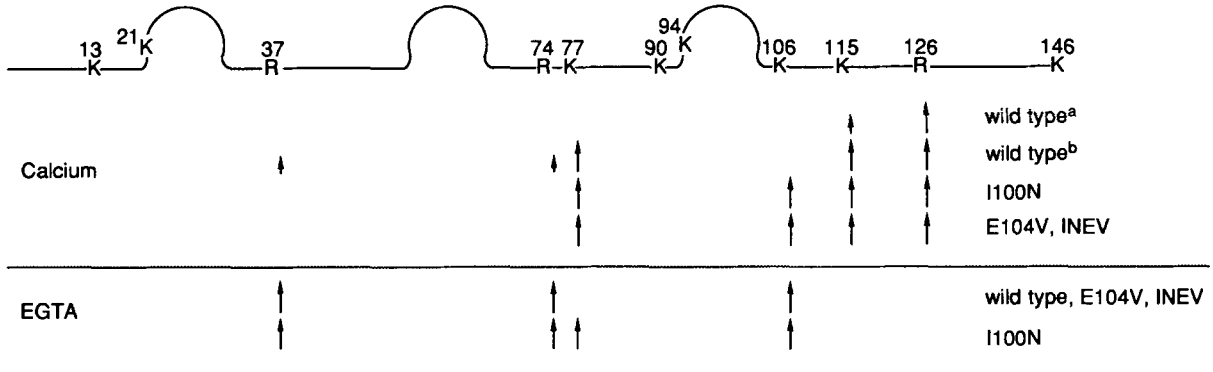

Fig. 6. Tryptic cleavage sites in wild-type and mutant forms of yeast calmodulin. Summary of the cleavage sites determined by mass spectrometric analysis of proteolytic mixtures and purified fragments as described in the Materials and methods and the legend for Figure 2. The weight ratio of trypsin to calmodulin and the length of digestion in the presence of Ca^{2+} were as follows: wild type[a], 1:500 for 30 min; wild type[b], 1:62.5 for 20 min; CamI100N, 1:62.5 for 2 min; CamE104V, 1:62.5 for 1 min; and CamINEV, a combination of two digestions 1:300 for 15 min and 1:625 for 15 min. For the digests done in the presence of EGTA, calmodulin was incubated for 30 min at a trypsin/calmodulin weight ratio of 1:500.

quentially from the C-terminus, as 107–146 was detected in each proteolytic mixture.

All forms of calmodulin had a similar level of susceptibility to trypsin in the presence of EGTA (data not shown). As determined by electrophoretic mobility and ESI mass spectrometry (Table 1), the digestion products were similar for all forms of calmodulin and included fragments 1–106 and 107–146. Fragments 1–126 or 1–115 were not observed.

Calmodulin-binding assay

To determine how the mutations alter the ability of calmodulin to interact with yeast proteins we used a modified gel overlay calmodulin-binding assay (Asselin et al., 1989; Wasco et al., 1989). Crude yeast extracts were subjected to electrophoresis on an SDS polyacrylamide gel; the proteins were transferred to a membrane and allowed to renature. Then, the membrane was probed with radioactive calmodulin. Usually such experiments are performed with iodinated calmodulin (Glenney & Weber, 1983). Although iodination introduces a large bulky group, the labeled calmodulin is assumed capable of binding to target proteins because it can activate several calmodulin-dependent enzymes. However, because calmodulin has different structural requirements for interaction with different proteins (Klee, 1988), the ability to activate several enzymes does not indicate that the labeled calmodulin can still bind to all targets. For this reason, we used ^{35}S-labeled yeast calmodulin, which was produced in *E. coli* and purified as described in the Materials and methods.

At 21 °C, in the presence of Ca^{2+}, wild-type calmodulin bound to four proteins in a yeast crude extract (Fig. 7). The apparent molecular weights of the four proteins are 67,000, 65,000, 60,000, and 58,000. Binding to these four proteins was barely detectable at a calmodulin concentration of 0.12 nM and was maximal at between 0.6 and 3.0 nM. Maximal binding occurred in less than 1 h. In the presence of EGTA, wild-type calmodulin did not bind detectably to any proteins when present at a concentration of 12 nM (data not shown). In the presence of Ca^{2+} or EGTA, at concentrations of calmodulin 20-fold larger than that required for maximal binding to the four proteins (60 nM), or after long incubations, calmodulin bound to many proteins with apparent molecular weights less than 31,000 (data not shown).

The mutant calmodulins were altered in their ability to bind to the four proteins that bind to wild-type calmodulin in a Ca^{2+}-dependent manner. At 21 °C, using 1.2 nM calmodulin, CamE104V and CamI100N both bound to the 67-kDa and the 65-kDa proteins but not to the two smaller proteins (Fig. 7). The double mutant, CamINEV, did not display strong binding to any of the four proteins but did bind at a low level to a protein of 67 kDa (Fig. 7). It is difficult to assess whether this represents specific

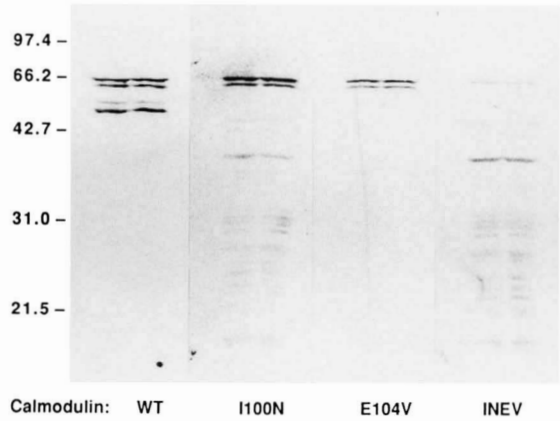

Fig. 7. Comparison of the ability of wild-type and mutant calmodulins to bind to proteins in a whole cell yeast extract. The binding assay was done as described in the Materials and methods in the presence of Ca^{2+}. Length of exposure of each blot to X-ray film was adjusted to correct for the differences in specific activity (5–15 cpm/fmol) between the different calmodulins. The molecular weight markers used were 97.4 kDa, rabbit muscle phosphorylase b; 66.2 kDa, bovine serum albumin; 42.7 kDa, ovalbumin; 31.0 kDa, bovine carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor.

binding to the Ca^{2+}-dependent target of 67 kDa or nonspecific binding to a protein of 67 kDa (see below). In either case, the presence of both mutations together disrupts the ability of Ca^{2+}-calmodulin to bind to proteins in a yeast extract to a greater extent than each individual mutation. Like wild-type calmodulin, at low concentrations, the mutant proteins did not detectably bind to proteins in the presence of EGTA. Changing the temperature of incubation to 4 °C or 37 °C did not alter the results (data not shown).

CamI100N and CamINEV also bound to at least 16 other proteins in the extract in the presence of Ca^{2+}. Most of these directly coincide with an abundant protein in the extract as determined by staining the blots with Ponceau S. Thus, although the binding appears to be largely Ca^{2+}-dependent, we believe it represents low affinity or nonspecific interactions.

To ensure that an inhibitor of binding was not present in the mutant preparations we probed membranes containing yeast extracts with mixtures of wild-type and mutant calmodulins. None of the mutant preparations interfered with wild-type binding to the four targets (data not shown).

Discussion

We have initiated a comparative study of the structure of yeast calmodulin based on the products of limited proteolysis with trypsin. In agreement with both their similarity in primary sequence (Davis et al., 1986) and their functional equivalence in vivo in yeast (Davis & Thorner, 1989; Ohya & Anraku, 1989), we conclude that yeast and

vertebrate calmodulin share a similar conformation. The tryptic fragments generated in the presence of Ca^{2+} or EGTA are remarkably conserved. However, a significant difference occurs near the fourth Ca^{2+}-binding loop at Arg-126. In vertebrate Ca_4^{2+}-calmodulin, Arg-126 is stable, whereas in yeast Ca_3^{2+}-calmodulin, Arg-126 is rapidly cleaved. The proteolytic susceptibility of Arg-126 is the first direct indication that the fourth putative Ca^{2+}-binding loop in yeast calmodulin does not bind Ca^{2+} with a high affinity. The result supports our previous analyses that loop 4 is ineffective in Ca^{2+} binding (Geiser et al., 1991). In the presence of EGTA, both yeast and vertebrate calmodulin are preferentially cleaved at residue 106. Calmodulins carrying mutations in the third Ca^{2+}-binding loop are unable to adopt the stable Ca^{2+}-bound conformation of that loop. This structural alteration interferes with binding to yeast Ca^{2+}-calmodulin targets.

The structure of vertebrate calmodulin has been examined extensively by biochemical and biophysical techniques (for review, see Forsen et al., 1986). Several early studies established the domain structure of vertebrate Ca_4^{2+}-calmodulin by identifying the products of limited digestion by trypsin (Walsh et al., 1977; Newton et al., 1984; Thulin et al., 1984). In the presence of Ca^{2+}, vertebrate calmodulin is predominantly cleaved at only 1 of the 14 basic residues, Lys-77 in the central helix. The protein is stabilized at least 10-fold when Ca^{2+} is bound (Walsh et al., 1977). In addition to Lys-77, Lys-115 is also susceptible to cleavage (C. Klee, pers. comm.) but is normally trimethylated. Thus, this cleavage is only observed in vertebrate calmodulin produced in bacteria, which lack the modification enzymes. A low level of cleavage also occurs at residues 37, 74, and 75 (Newton et al., 1984; Thulin et al., 1984).

We have reinterpreted the previous data on the limited proteolysis of vertebrate calmodulin (Walsh et al., 1977; Newton et al., 1984; Thulin et al., 1984) in view of the crystal (Babu et al., 1988) and solution structures (Ikura et al., 1991) now available (see Kinemage 2). The level of susceptibility of the lysine and arginine residues in higher eukaryotic Ca_4^{2+}-calmodulin correlates with the regularity of the secondary structure surrounding the residue. Thus, one of the most susceptible residues, Lys-115, is at the bend between two EF hands. The other susceptible residue, Lys-77, is in a region of the central helix that appears distorted in the crystal structure (Babu et al., 1988) and flexible in the solution structure (Ikura et al., 1991). Residues that are not cleaved or are cleaved at a low level are found in α-helices. The exceptions are the two lysines within the Ca^{2+}-binding loops. Although not in a regular secondary structure, these two residues are not cleaved even after extensive digestion because they are between acidic residues (Watterson et al., 1980).

In the presence of 1 mM $CaCl_2$, yeast calmodulin produced in *E. coli* is cleaved sequentially by trypsin at Arg-126, then at Lys-115, and finally at Lys-77, with a low level of cleavage at residues 37 and 74. Thus, yeast calmodulin is similar to vertebrate calmodulin in that it contains a relatively stable N-terminal domain and is cleaved at residues 77 and 115. Furthermore, the susceptibility of the basic residues is similar to those in vertebrate calmodulin except for Arg-126, which is surprisingly susceptible to proteolytic attack. At a trypsin/calmodulin ratio of 1:62.5, yeast Ca_3^{2+}-calmodulin is cleaved almost to completion at Arg-126 in only 5 min. In contrast, although Arg-126 is conserved in vertebrate calmodulin, cleavage there is not detectable even after 40 min at a ratio of 1:60 (Newton et al., 1984).

In vertebrate Ca_4^{2+}-calmodulin, the fourth Ca^{2+}-binding loop forms a helix–loop–helix structure termed an EF hand. Arg-126 in vertebrate calmodulin is stable to proteolysis presumably because it is in an α-helix, which precedes the Ca^{2+}-binding loop (Babu et al., 1988). The unusual accessibility of Arg-126 in yeast Ca_3^{2+}-calmodulin suggests that the structure of the surrounding region is irregular and is not an α-helix, thus suggesting that the fourth loop is not folded into an EF hand even at 1 mM Ca^{2+}. If the fourth loop does not bind Ca^{2+}, then the accessibility of Arg-126 to trypsin should be similar in apocalmodulin and Ca^{2+}-calmodulin. However, in apocalmodulin, cleavage at residue 106 occurs too quickly to detect cleavage at Arg-126 even if it occurs. In agreement with our conclusion that the fourth loop is inactive, Matsuura and coworkers (1991) recently showed that a mutant yeast calmodulin in which the fourth loop is deleted still binds three calcium ions as does the intact protein. An inactive fourth loop is the main structural difference detected between yeast and vertebrate calmodulin and may be the main cause of the decreased affinity of yeast calmodulin for mammalian targets.

ESI mass spectrometry permitted the rapid and reliable recognition of 10 proteolytic fragments of calmodulin. For the five large major fragments 1–126, 1–115, 1–106, 1–90, and 1–77, when the fragment was detected by ESI mass spectrometry it was also detected on an SDS polyacrylamide gel and vice versa. The smaller fragments were not always well resolved on the SDS gel, and thus a correlation was more difficult to establish. Our mixing experiment did not suggest a suppression or bias that might prevent detection of the constituents important for the conclusions of this study. Others have noted that the molar responses for different proteins vary widely (C.G. Edmonds, J.A. Loo, & R.D. Smith, unpubl.). However, the analysis of proteolytic mixtures by ESI mass spectrometry reveals peptides not seen by other mass spectrometric ionization methods (Chowdhury et al., 1990; Edmonds et al., 1991).

As another assay for the structural differences between yeast Ca_3^{2+}-calmodulin and apocalmodulin, a gel overlay assay was used to characterize the binding of calmodulin to proteins in a yeast crude extract in the presence of

Ca^{2+} and EGTA. The assay exploits the fact that calmodulin-binding domains are small and contiguous and thus rapidly renature after removal of SDS (Glenney & Weber, 1983). Wild-type yeast calmodulin binds to four proteins in a Ca^{2+}-dependent manner. The apparent molecular weights of the four yeast proteins (67,000, 65,000, 60,000, and 58,000) suggest that they may correspond to the two yeast Ca^{2+}-dependent protein phosphatases with predicted molecular weights of 68,503 and 63,003 (Cyert et al., 1991) and the two yeast Ca^{2+}–calmodulin-dependent protein kinases with apparent molecular weights of 56,000 and 50,000 (Ohya et al., 1991; Pausch et al., 1991). Even at high concentrations, yeast calmodulin does not bind to the four proteins in the presence of EGTA, although binding to many proteins with apparent molecular weights at 31,000 or below could be detected whether or not free Ca^{2+} was available. Using a similar assay, Liu and coworkers (1990) obtained similar results. A high concentration of ^{125}I-labeled bovine calmodulin (10 nM compared to 1.2 nM) was used, and thus both high affinity and low affinity interactions were detected.

The analysis was extended to examine the structural alterations caused by mutations that together confer a temperature-sensitive phenotype in yeast. The two mutations, E104V and I100N, are both in the third Ca^{2+}-binding loop. We assayed the effects of the mutations on (1) the susceptibility of calmodulin to proteolysis by trypsin and (2) the ability of calmodulin to bind to proteins in a yeast crude extract. Mutant calmodulins carrying one or both mutations are more sensitive to proteolysis by trypsin than wild-type calmodulin, although neither mutation introduces a lysine or an arginine. In the presence of Ca^{2+}, the mutant proteins are degraded to the size of half molecules 10–15 times faster than wild-type calmodulin. Furthermore, in the mutant calmodulins, Lys-106, located two residues from the third loop, is accessible to trypsin in both the presence and absence of Ca^{2+}. In contrast, this site is accessible to proteolytic cleavage in wild-type calmodulin only in the absence of Ca^{2+}. These results strongly suggest that both mutations prevent formation of the stable Ca^{2+}-bound conformation in the region surrounding the third Ca^{2+}-binding loop.

In several cases it has been shown that alterations that increase the thermolability of a protein also increase the susceptibility of that protein to proteases. The simplest explanation for this correlation is that proteases show enormous preferences for denatured proteins (Pace & Barrett, 1984). The most thermolabile proteins are more susceptible to proteolysis because a greater portion of the protein is in the unfolded state. For example, the most thermolabile variants of the N-terminal domain of the λ repressor have the shortest half-lives in *E. coli* (Parsell & Sauer, 1989). In addition, the C-terminal domain of vertebrate calmodulin, which has a melting temperature that is 30 °C lower in the presence of EGTA than in the presence of Ca^{2+} (Tsalkova & Privalov, 1985), is at least 10-fold more susceptible to proteolysis by trypsin in the presence of EGTA. The increased susceptibility of the mutant yeast calmodulins to trypsin in the presence of Ca^{2+} and the exposure of Lys-106 near the third Ca^{2+}-binding loop suggests that the mutations I100N and E104V increase the thermolability of calmodulin by interfering with Ca^{2+}-binding in the third loop.

As assayed by the level of susceptibility to trypsin, mutant proteins carrying either mutation alone are not distinguishable from the double mutant protein. Thus, as discussed, all three mutant proteins are predicted to be thermolabile. Yet, only both mutations together confer temperature sensitivity; yeast strains carrying only the I100N mutation or the E104V mutation are viable at 37 °C (T.N. Davis, unpubl.). That CamINEV has more severe structural alterations than either CamI100N or CamE104V was revealed by the calmodulin-binding assay. CamINEV was defective in binding to all four of the proteins detected by the gel overlay assay; whereas CamI100N and CamE104V were only defective in binding to two of the four. How these structural alterations affect interaction with the as yet unidentified Ca^{2+}-independent targets (Geiser et al., 1991) remains to be studied.

In conclusion, as assayed by susceptibility to trypsin, yeast calmodulin has a conformation similar to that of vertebrate calmodulin with the exception that the fourth Ca^{2+}-binding loop in yeast calmodulin does not bind Ca^{2+}. The mutations I100N and E104V disrupt the Ca^{2+}-bound conformation in the third loop and alter the ability of calmodulin to interact with yeast proteins in a Ca^{2+}-dependent manner.

Materials and methods

Strains and plasmids

Proteins were produced in *E. coli* strains SB1 (Geiser et al., 1991) and GM1 (Coulondre & Miller, 1977).

All plasmids constructed in this study are listed in Table 2. Plasmid pTD35 was constructed as follows. The 1.1-kb *Bgl*II fragment carrying the yeast calmodulin gene (*CMD1*) was excised from plasmid pTD30 (Davis et al., 1986). *Bam*HI linkers were added by ligation, and then excess linkers were removed. The fragment then was ligated into the *Bam*HI site of the replicative form of phage mp9. An *Nco*I site was created at the initial ATG of *CMD1* in plasmid pTD35 by site-directed mutagenesis (Kunkel et al., 1987) using the Bio-Rad Muta-Gene kit. Mutant transformants were identified by restriction digest analysis. The presence of the mutation was further confirmed by DNA sequencing (Tabor & Richardson, 1987). The resulting mutant plasmid was then digested with *Sna*BI, which cuts 3′ of the calmodulin gene. After ligation of *Nco*I linkers to the *Sna*BI end, the *Nco*I fragment containing the entire calmodulin gene was excised and ligated into pMW1 to create pSB4. Plasmid pMW1

Table 2. *Plasmids used in this study*

Plasmid name	Parent plasmid	Relevant markers
pTD35	mp9	*CMD1*[a]
pMW1	pKK233-2	*trc* promoter, f1 origin
pSB4	pMW1	*trc* promoter, S1A *CMD1*, f1 origin
pSB5	pSB4	*trc* promoter, S1A *CMD1*, f1 origin
pSB7	pSB5	*trc* promoter, S1A, I100N, E104V *CMD1*, f1 origin
pSB8	pSB5	*trc* promoter, S1A, I100N *CMD1*, f1 origin
pSB9	pSB5	*trc* promoter, S1A, E104V *CMD1*, f1 origin
pJG28	pSB5	*trc* promoter, *CMD1*, f1 origin
pSB11	pJG28	*trc* promoter, I100N *CMD1*, f1 origin
pSB12	pJG28	*trc* promoter, E104V *CMD1*, f1 origin
pSB13	pJG28	*trc* promoter, I100N, E104V *CMD1*, f1 origin

[a] *CMD1* is the gene encoding yeast calmodulin.

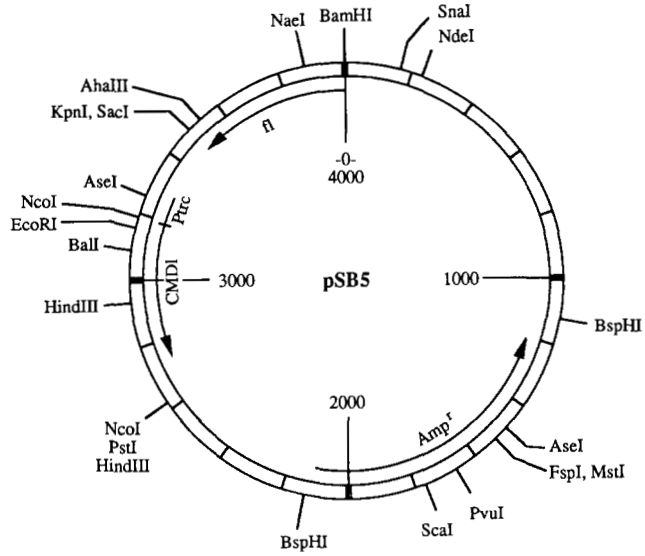

Fig. 8. Bacterial expression plasmid pSB5. Plasmid pSB5 is a derivative of plasmid pKK233-2 (Pharmacia) and contains the intact *CMD1* gene with an *Nco*I site at the ATG under the control of the bacterial *trc* promoter. Only restriction enzyme sites that have a 6-base recognition sequence and are present in the plasmid three or fewer times are shown.

is a version of bacterial expression vector pKK233-2 (Pharmacia) in which copy number control was destroyed, and a *Bam*HI-*Eco*RI fragment containing the f1 origin of replication from pUC-f1 (Pharmacia) was inserted in place of the *Eco*RI-*Pvu*II fragment pKK233-2. Plasmid pSB5 was constructed by removing one of two *Eco*RI sites in pSB4; the remaining *Eco*RI site is in the calmodulin gene (Fig. 8). Plasmids pSB7, pSB8, and pSB9 were constructed by replacing the wild-type calmodulin *Eco*RI-*Pst*I fragment in pSB5 with *Eco*RI-*Sna*BI mutant calmodulin fragments containing both I100N and E104V, just I100N, or just E104V, respectively. The *Eco*RI-*Sna*BI calmodulin fragments containing either the I100N mutation or the E104V mutation were isolated from plasmid pTD35 mutagenized by site-directed mutagenesis. The mutant *Eco*RI-*Sna*BI calmodulin fragment with both the E104V and I100N mutations was obtained from plasmid pTD79, which is a randomly mutagenized derivative of plasmid pTD59 (Geiser et al., 1991) (see below). Plasmids pSB5, 7, 8, and 9 were used to produce the calmodulins used for tryptic digests except in Figures 4 and 5 (see below). The presence of an *Nco*I site at the beginning of the calmodulin gene changes the second amino acid residue from a serine to an alanine.

Plasmids pJG28, pSB11, pSB12, and pSB13 were used to produce the ^{35}S-labeled wild-type and mutant calmodulins used in the binding assay and for the experiments described in Figures 4 and 5. Plasmid pJG28 is a derivative of pSB5. It was constructed by removing the *Nco*I site at the start of the calmodulin gene by site-directed mutagenesis. Thus, pJG28 encodes wild-type calmodulin. In plasmids pSB11, 12, and 13, the *Eco*RI-*Sca*I fragment of pJG28 carrying wild-type calmodulin was replaced with the *Eco*RI-*Sca*I fragment from pSB8, 9, and 7, respectively. For all plasmids, the presence of mutations was confirmed by DNA sequencing.

Isolation of a temperature-sensitive calmodulin mutant

Isolation of a temperature-sensitive calmodulin mutant will be described in detail elsewhere (T.N. Davis, unpubl.). Briefly plasmid pTD59 (a plasmid that can replicate in yeast or *E. coli* and carries a centromere and the *CMD1* gene) (Geiser et al., 1991) was subjected to random mutagenesis and transformed into yeast strain TDY55-5D (pTD56) (Geiser et al., 1991). Cells carrying a temperature-sensitive calmodulin (CamINEV) were identified by the plasmid shuffling procedure described previously (Geiser et al., 1991). Two mutations were required to confer a temperature-sensitive phenotype. The mutations were identified by sequencing the mutant *CMD1* gene (Tabor & Richardson, 1987). One mutation changes isoleucine 100 to an asparagine (I100N), and the other changes glutamic acid 104 to a valine (E104V).

Protein purification

Escherichia coli strains carrying the appropriate plasmids were grown at 37 °C in LB medium (Miller, 1972) containing 100 μg/mL ampicillin. At a cell density between 10 and 20 Klett units, calmodulin production was in-

duced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. When the cultures reached 200 Klett units, cells were collected by centrifugation, washed twice in 50 mM Tris/HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride, and 1 mM $MgCl_2$ and then lysed using a French press at 16,000 psi. Cellular debris was removed by ultracentrifugation for 30 min at 200,000 × *g*. Wild-type yeast calmodulin was purified essentially as described (Ohya et al., 1987). Briefly, EDTA was added to 2 mM to the clarified lysate, which was then passed over a phenyl-Sepharose column to remove proteins that bound in a Ca^{2+}-independent manner and lipids (100 mg protein/3 mL [2/3] phenyl-Sepharose slurry). The flow through from this column was brought to 10 mM $CaCl_2$, 1.0 M ammonium sulfate, and loaded on a second phenyl-Sepharose column (2 mL packed phenyl-Sepharose/1 mg calmodulin). This second column was washed with 10 column volumes of 50 mM Tris/HCl, pH 7.5, containing 5 mM $CaCl_2$, and 0.1 M ammonium sulfate, and then the calmodulin was eluted with 50 mM Tris/HCl, pH 7.5, containing 50 mM EGTA and 0.1 M ammonium sulfate. The fractions containing calmodulin were pooled, dialyzed against 0.1 M ammonium bicarbonate, and lyophilized. For purification of the mutant calmodulins (CamE104V, CamI100N, and CamINEV), the procedure was further modified as these proteins do not bind to phenyl-Sepharose as well as wild-type calmodulin. The wash buffer contained 0.25 M instead of 0.1 M ammonium sulfate, and the elution buffer contained 100 mM instead of 50 mM EGTA. CamE104V, CamI100N, and CamINEV were further purified through anion exchange chromatography. Proteins were eluted from a Mono-Q FPLC (Pharmacia) column with a linear 0.05–0.65 M NaCl gradient in 50 mM sodium phosphate buffer, pH 6.5, dialyzed as above and then lyophilized. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce) with bovine serum albumin as a standard.

For purification of ^{35}S-labeled wild-type and mutant calmodulins, the above procedure was slightly modified. The cells were grown in 30 mL of M9 medium (Miller, 1972), containing 1.0 mM $MgCl_2$ instead of $MgSO_4$, 0.4% glucose, 1 μg/mL thiamine, and 10 mCi carrier-free $Na^{35}SO_4$ (NEN), to 10–20 Klett units and induced as described above. The cultures ceased growth at 65 Klett units and were then harvested and lysed as described (Geiser et al., 1991). The ^{35}S-labeled calmodulin was purified by chromatography on phenyl-Sepharose as described above. The calmodulin had a specific activity of approximately 10 cpm/fmol.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described (Laemmli, 1970). For calmodulin gels, the bisacrylamide:acrylamide weight ratio was 38:2, and the gel concentration was 14%. The nondenaturing gel system used was the same except that it did not contain SDS. For separating proteins in whole cell yeast extracts, the bisacrylamide:acrylamide weight ratio was 39:1, and the gel concentration was 11%. Gels were stained with Coomassie Brilliant Blue R-250.

Proteolysis

Trypsin (251 units/mg protein) treated with TPCK was obtained from Worthington Biochemicals. Tryptic digests were performed at room temperature in 20 mM ammonium bicarbonate buffer (pH 8.0) containing 1 mM $CaCl_2$ or 1 mM EGTA. The calmodulin concentration was 1 mg/mL. The weight ratio of calmodulin to trypsin varied (refer to Figs. 2–6). Proteolysis was started by adding freshly diluted trypsin and terminated by adding soybean trypsin inhibitor (1.4 mg soybean trypsin inhibitor/mg trypsin). When comparing the proteolytic susceptibility of the different calmodulins, the digestions were done simultaneously.

Purification of tryptic fragments

Wild-type calmodulin was digested for 20 min in 1 mM $CaCl_2$ at a weight ratio of trypsin to calmodulin of 1:62.5. The three major fragments produced were purified by reverse-phase HPLC, using a Delta-Pak C4 column (Millipore: 3.9 mm × 30 cm, 300 Å pore size). All HPLC eluent solutions were purified by Millipore filtration (0.45 μM) and degassed before use. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid in acetonitrile. The gradient used was 0–35% buffer B in 2 min and then 35–55% buffer B in 80 min.

N-terminal sequence analysis

The three tryptic fragments produced after a partial digest of wild-type calmodulin in the presence of Ca^{2+} were electroblotted onto a PVDF membrane as described (LeGendre & Matsudaira, 1988) and then subjected to Edman degradation using an Applied Biosystems Model 470 gas phase sequencer equipped with an on-line Model 120A phenylthiohydantoin analyzer.

ESI mass spectrometry

The success of the mass spectrometry depends on the samples being substantially salt-free. Thus, proteolytic mixtures were desalted exhaustively using a Centricon-3 microconcentrator (Amicon). All fragments less than 3,000 Da were lost during the desalting procedure. Digests were then analyzed by ESI mass spectrometry using a TAGA 6000E tandem quadrupole instrument (SCIEX, Thornhill, Ontario, Canada) equipped with ion source and atmosphere/vacuum interface previously described

(Smith et al., 1988, 1990). Solutions of analyte at 1.0 mg/mL in 5% acetic acid solution were delivered at 0.5 μL/min to the ion source operating under standard conditions. Data were acquired at 0.2-*m/z* intervals in scanning the mass spectrometer; five spectra were averaged and the centroid of each ion was selected. The mixed spectra were deconvoluted by inspection and the relative molecular masses of the constituents calculated according to principles already described (Mann et al., 1989; Edmonds & Smith, 1990). These data were compared to calculated masses of all possible tryptic fragments and assignments made with mass measurement errors generally less than 0.05%.

Calmodulin-binding protein assay

Whole cell yeast extracts were made as described (Wright et al., 1989). Proteins (50 μg) were separated on an SDS polyacrylamide gel and then electrophoretically transferred to an Immobilon membrane (Millipore) using a Transblot semidry apparatus following the instructions provided by the manufacturer (Bio-Rad). The transfer buffer was 48 mM Tris and 39 mM glycine. After transfer, the proteins were renatured by washing the Immobilon membrane for a total of 30 min (three changes) in buffer C (20 mM HEPES, pH 7.2, 200 mM NaCl), blocked for a minimum of 1 h in buffer C with 3% BSA and 0.05% Tween-20, and then probed at 21 °C for 1 h with 1.2 nM wild-type or mutant ^{35}S-labeled calmodulin (10 cpm/fmol) in buffer D (buffer C with 1 mM $CaCl_2$, 0.05% Tween 20, 0.1% BSA, and 0.1% gelatin) or buffer E (buffer D with 1 mM EGTA instead of 1 mM $CaCl_2$). The membrane was then washed two times each for 2 min and then three times each for 10 min in buffer D or E, air dried, dipped in 7% 2,5-diphenyloxazole in acetone, air dried, and exposed to X-ray film (Kodak).

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