Calmodulin−Peptide Interactions: Apocalmodulin Binding to the Myosin Light Chain Kinase Target-Site†

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Received January 21, 2000; Revised Manuscript Received April 3, 2000

ABSTRACT: Noncovalent binding of the synthetic peptide RS20 to calmodulin in the presence of calcium was confirmed by electrospray ionization coupled with Fourier transform ion cyclotron resonance mass spectrometry to form a complex with a 1:1:4 calmodulin/RS20/calcium stoichiometry. There was no evidence for formation of a calmodulin−RS20−Ca_2 species. The absence of calmodulin−RS20−Ca_2 would be consistent with models in which the two globular domains are coupled functionally. There was evidence that calmodulin, RS20−calmodulin *without* associated calcium, and calmodulin−RS20−Ca_4 existed together in solution, whereas calmodulin−calcium complexes were absent. It is proposed that calcium binding to form the calmodulin−RS20−Ca_4 complex occurs *after* an initial RS20−calmodulin binding event, and serves to secure the target within the calmodulin structure. The binding of more than one RS20 molecule to calmodulin was observed to induce unfolding of calmodulin.

Calmodulin is a relatively small, acidic, heat-stable protein involved in diverse calcium-mediated interactions in eukaryotic cells (*1, 2*). The structure of calmodulin has been highly conserved throughout evolution with a sequence closely homologous to that of other calcium-binding proteins such as troponin C and the parvalbumins, suggesting a family of proteins that have evolved from a single ancestral calcium-binding protein. Although troponin C and parvalbumin are tissue specific and believed to have evolved for specific functions, calmodulin is ubiquitous and has been found in all tissues investigated to date.

Calmodulin has the shape of a dumb-bell with two globular domains, each containing two high-affinity calcium-binding sites (EF-hand sites), i.e., helix−loop−helix motifs connected by a solvent-exposed flexible linker (*3*−*9*). Calcium binding to calmodulin has been shown to be associated with conformational changes and exposure of hydrophobic areas in each of the globular lobes. These lobes constitute important binding sites for most target proteins (*10, 11*). It has been assumed that it is exposure of the hydrophobic residues as a result of calcium binding that subsequently promotes binding of the target protein. In the presence of calcium, calmodulin is able to interact strongly with, and regulate selectively, a remarkable variety of proteins which have little sequence homology in their calmodulin-binding region, although most do have a tendency to form basic amphiphilic α-helices (*12*). Calmodulin is also able to interact with target proteins such as neuromodulin and brush border myosin in its calcium-free form (*13*), and certain target proteins are known to be activated by calmodulin in both the absence and the presence of calcium (*14*). Calmodulin interactions with target proteins have been shown to be mediated by both hydrophobic and electrostatic forces, with the predominant interactions occurring between the N-terminal domain of calmodulin and the C-terminal end of the target, and vice versa (*9, 15*).

In addition to the four main calcium-binding sites, calmodulin has been found to possess auxiliary sites for cation binding (*16, 17*). Saturation of calmodulin with calcium (1 mM) has been shown to lead to the binding of up to at least 10 calcium ions. This is consistent with the low-resolution electrospray ionization (ESI[1]) mass spectrometry results of Gross and co-workers (binding of one to seven Ca^{2+} ions, *18*), but in contrast to those of Veenstra et al. (*19*) and Loo and co-workers (*20*) who both reported that calmodulin binds a maximum of four calcium ions at 1 mM calcium acetate. Auxiliary sites play a role in the binding of cations such as magnesium or zinc and, therefore, participate in the tuning of calmodulin.

Electrospray ionization mass spectrometry has been exploited at low resolution to study the binding properties of calmodulin with peptides, such as melittin and calmodulin-dependent protein kinase II (*18*−*21*). Building on the investigations of Haiech and colleagues (*17, 22*), we have employed high-resolution ESI−Fourier transform ion cyclotron resonance (FTICR) (*23*) to probe at the microscopic level the equilibria and sequence of binding events occurring between calmodulin and RS20 (a synthetic peptide analogue derived from the phosphorylation site of smooth-muscle myosin light chain kinase (MLCK)) in the presence and absence of calcium (and magnesium) ions.

† This work was supported by grants from the EPSRC (GR/K42042), the BBSRC (42/B07297 and 88/9708907), and Zeneca. The University of Warwick FTICR mass spectrometry facility is a UK National Facility supported by the EPSRC.

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[1] Abbreviations: ESI, electrospray ionization; FTICR, Fourier transform ion cyclotron resonance; MLCK, myosin light chain kinase.

10.1021/bi000139m CCC: $19.00
Published on Web 05/24/2000

The calmodulin-binding domain of MLCK was the first of such domains to be identified, and peptides derived from the amino acid sequence of this domain have been characterized as inhibitors of calmodulin activation. In most cases, these peptides have the same binding affinity (picomolar to low nanomolar) and stoichiometry as those associated with the target enzyme as a whole (*24*−*27*). Peptides based on the binding domains of target proteins have allowed investigations to be made without the complications associated with the high molecular weights of most target enzymes. Until recently, it was believed that MLCK and MLCK-derived peptides were able to interact with calmodulin only in the presence of calcium. We have reported that significant binding of RS20 to calmodulin also occurs in the absence of calcium (*28*). On the basis of a combination of isothermal titration calorimetry and differential scanning calorimetry, Tsvetkov et al. *(29)* have confirmed interaction between calmodulin and RS20 in the absence of calcium. They have proposed that the peptide interacts with the C-terminal lobe of apocalmodulin only; i.e., the peptide does not bridge the C- and N-terminal lobes. On the basis of our high-resolution ESI−FTICR results, we hypothesize that the apocalmodulin−RS20 complex is an intermediate in the formation of the calmodulin−RS20−Ca_4 complex, which is a species recognized to be physiologically relevant. We have found no evidence to support the existence of a calmodulin−RS20−Ca_2 complex, which would have been analogous to the calmodulin−M13−Ca_2 species proposed by Bayley et al. (*30*).

EXPERIMENTAL PROCEDURES

Protein Synthesis and Purification. DNA-encoded calmodulin was obtained as previously described and purified by column chromatography (*31*, *32*). The purity of the protein was checked by SDS−PAGE and high-pressure capillary electrophoresis, and it was found to be approximately 99% pure. Ultrapure water (Elga system) and plastic ware which had been washed in 1 N HCl were used to minimize metal cation and other contamination. Calmodulin (2.5 mg) was dissolved in 2.0 mL of ammonium acetate (5 mM, pH 5.9) and purified over a desalting PD 10 column (Pharmacia Uppsala) which had been previously equilibrated with ammonium acetate. The calmodulin concentration in the fraction used was determined by UV absorption on a Jasco V-550 spectrophotometer using a molar extinction coefficient for calmodulin of $\epsilon_{280\ nm} = 1560\ M^{-1}\ cm^{-1}$ (*22*).

Calmodulin−RS20 Sample Preparation. The RS20 peptide was synthesized and purified (greater than 99% pure) as previously described *(33, 34)*. A stock solution of the peptide was prepared by dissolving a 1.3-mg sample of lyophilized peptide in 565 μL of ammonium acetate (5 mM, pH 5.9) giving a concentration of 1.0 mM. Small aliquots of peptide stock were added to calmodulin solutions to achieve desired calmodulin to RS20 molar ratios. The calmodulin concentration was typically 25 μM (0.4 mg/mL), and peptide concentrations were varied from approximately 40 μM to several hundred micromoles. Where necessary, small aliquots (10 μL) of a concentrated $CaCl_2$ or $MgCl_2$ (Aldrich) stock were added to calmodulin−peptide solutions to give the required $CaCl_2$ or $MgCl_2$ concentrations. The use of physiologically relevant conditions is recognized to be important for maintaining the integrity of noncovalent complexes; the use of organic solvents and acids (often used in more routine ESI mass spectrometry) has been avoided in the experiments reported here.

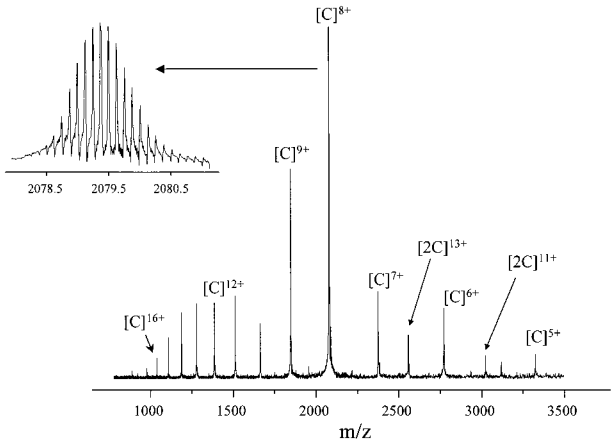

FIGURE 1: ESI−FTICR mass spectrum of calmodulin in ammonium acetate buffer, 5 mM, pH 5.9. Inset shows the isotopically resolved pattern of the 8^+ charge-state.

Electrospray Ionization−Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. Mass spectrometry measurements were made using an FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a passively shielded 9.4 T superconducting magnet (Magnex Scientific Ltd., Abingdon, UK), a cylindrical infinity ICR cell with 0.06-m diameter, and an external ESI source (Analytica of Branford, Branford, CT). This FTICR instrument has been described previously (*35*, *36*). The ESI source was equipped with a Pyrex capillary which was coated on both ends with platinum paint. The voltages on the nozzle and the skimmer were kept appropriately low (typically 60 and 3 V, respectively). Carbon dioxide was used as the drying gas in the electrospray source, and its temperature was carefully controlled. The background pressure in the ICR analyzer cell was typically below 2×10^{-10} mbar.

Differential Scanning Calorimetry. Microcalorimetric measurements were carried out on a MicroCal VP-DSC instrument in 0.51 cells at a heating rate of 1 K/min. Protein concentration varied from 0.8 to 1.1 mg/mL. The heating curves were corrected for an instrumental baseline, based on heating the solvent used for protein solution. The calorimetric denaturation enthalpy (ΔH_{cal}) and the partial molar heat capacity of the protein (C_p) were determined as described elsewhere, assuming the relative molecular mass of calmodulin to be 16 628 and the partial specific volume to be 0.72 $cm^3\ g^{-1}$ *(38)*. To analyze functions of excess heat capacity, the MicroCal Origin software was used. The calorimetric denaturation enthalpy values are accurate to within ±8%, and C_p values at 20 °C are accurate to within ±1.5 $kJ\ K^{-1}\ mol^{-1}$. The errors in the parameters of individual transitions obtained by deconvolution of complex endotherms did not exceed ±10% for transition enthalpy or ±0.6 °C for transition temperature.

RESULTS AND DISCUSSION

Calmodulin in Buffer. Figure 1 shows the ESI−FTICR mass spectrum obtained for calmodulin in 5 mM ammonium acetate buffer, pH 5.9, in the absence of an organic solvent. Peaks in Figure 1 have been assigned to protonated mol-

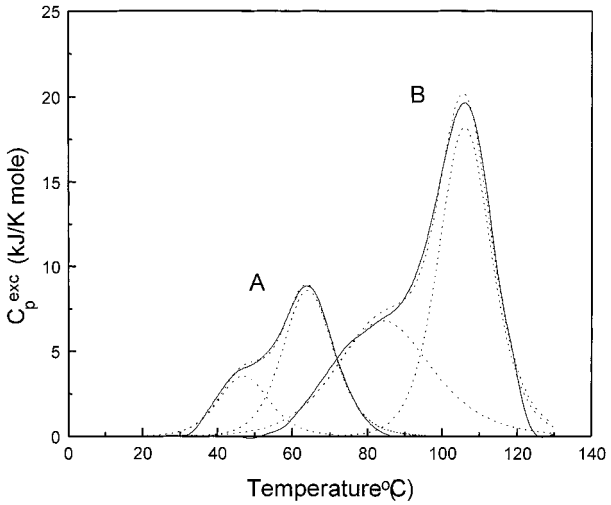

FIGURE 2: Deconvolution of the transition excess heat capacity of calmodulin (A) and calcium-saturated calmodulin (B) in 10 mM ammonium acetate buffer at pH 6.0. Solid lines, experimental results; dotted lines, deconvolution peaks and their sum.

ecules. Thus, for example, $[C]^{8+}$ represents [calmodulin + $8H]^{8+}$ where calmodulin is the neutral protein. The experimentally determined monoisotopic mass of calmodulin was 16 616.84 ± 0.02 Da, which agrees with the theoretical mass of the isotopically pure protein obtained from the sequence (16 616.821 Da) to within experimental error.

As reported previously (*23*), there were at least two charge-distribution patterns in the ESI−FTICR mass spectrum of calmodulin indicating the presence of at least two different protein conformations. The higher charge-state distribution, centered around the 12^+ charge-state, was similar to that observed for solutions sprayed from methanol or acetonitrile/water mixtures (*23*) and is proposed to correspond to more open (i.e., unfolded) calmodulin conformations. The lower charge envelope, exhibiting a maximum corresponding to eight attached protons, is attributed to the presence of more compact (i.e., folded) calmodulin conformations, only observable under the more physiologically relevant buffer conditions. Correlations between charge-state distribution patterns observed in ESI mass spectra and the equilibrium of protein conformations which exist in solution have been illustrated and established for other proteins (*37, 39*−*42*).

Signals corresponding to dimeric calmodulin species (labeled $[2C]^{n+}$) were observed in buffered aqueous solutions as previously reported (*23*).

To determine whether there was any significant influence of the ammonium acetate buffer on calmodulin's structure in the absence of salt, differential scanning calorimetric experiments were carried out as a control. Temperature dependences of the partial molar heat capacities of both apocalmodulin and its calcium-saturated form in 10 mM ammonium acetate buffer were found to be very similar to those in 10 mM cacodylate buffer and 150 mM KCl as described previously *(29)*. Deconvolution of melting curves on two two-state transitions (Figure 2) gave almost the same denaturation temperatures and enthalpies for calmodulin in ammonium acetate buffer as in cacodylate buffer in the presence of salt (see Table 1). The partial molar heat capacity of apocalmodulin and calcium-saturated calmodulin at 20 °C was 31.9 kJ K^{-1} mol^{-1}, which is close to the value in 10 mM cacodylate, 150 mM KCl buffer (i.e., 31.4 kJ K^{-1} mol^{-1})

Table 1: Comparison of the Scanning Calorimetry Parameters of Temperature-Induced Denaturation of Calmodulin and Calcium-Saturated Calmodulin at pH 6.0 in 10 mM Ammonium Acetate and at pH 7.5 in 10 mM Cacodylate, 150 mM KCl

sample	buffer	ΔH_{cal}[a]	T_t^1[b]	ΔH_t^1[b]	T_t^2[c]	ΔH_t^2[c]
calmodulin	10 mM ammonium acetate	332	45.3	145	64.0	193
calmodulin[d]	10 mM cacodylate, 150 mM KCl	361	47.4	147	65.0	214
calmodulin−Ca^{2+}	10 mM ammonium acetate	352	78.5	127	105.5	251
calmodulin−Ca^{2+} [d]	10 mM cacodylate, 150 mM KCl	336	80.2	129	103.4	250

[a] ΔH_{cal}, total calorimetric enthalpy of denaturation. [b] T_t^1 and ΔH_t^1, temperature and enthalpy of first transition (C-terminal lobe unfolding, see ref 38). [c] T_t^2 and ΔH_t^2, temperature and enthalpy of second transition (N-terminal lobe unfolding, see ref 38). [d] Reference 29.

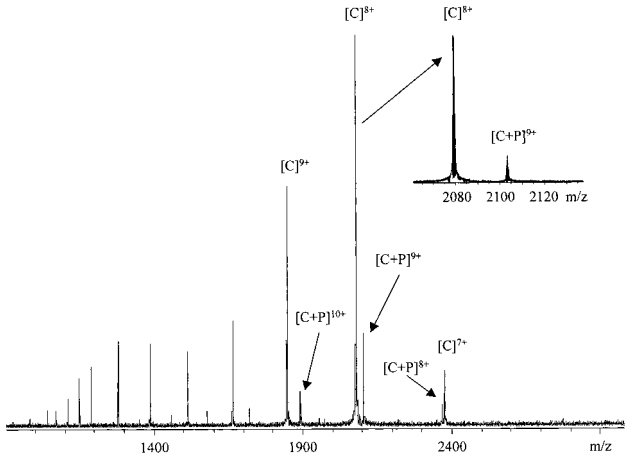

FIGURE 3: ESI−FTICR mass spectrum of calmodulin with RS20 peptide (concentration ratio 1:1.5) in ammonium acetate buffer, 5 mM, pH 5.9, in the absence of calcium. Inset shows the expansion of the 8^+ calmodulin charge-state. P represents RS20.

(38). Thus, it can be concluded that there are no dramatic conformational changes of calmodulin under the buffer conditions used for mass spectrometry, as compared to standard biochemical conditions.

RS20 in Buffer. In the ESI−FTICR mass spectrum (not shown) obtained for RS20 in 5 mM ammonium acetate buffer, pH 5.9, the most abundant RS20 species found was that with three attached protons ($[RS20 + 3H]^{3+}$). The experimentally determined monoisotopic mass of RS20 was 2293.293 ± 0.01 Da, in agreement with the theoretical mass of the isotopically pure peptide obtained from the sequence (2293.299 Da) to within experimental error.

Calmodulin−*RS20 Interaction.* A positive ESI−FTICR spectrum obtained following the addition of RS20 to a calcium-free solution of calmodulin in buffer is shown in Figure 3. The concentration ratio was 1.5:1 peptide/calmodulin for this spectrum. The presence of several new peaks, as compared to the spectrum of calmodulin alone (Figure 1), indicated the presence of a 1:1 calmodulin−RS20 complex (without any associated calcium ions). For example, the signal at $m/z = 2103.46$ represented a species with a charge-state of 9^+ and gave the derived mass of

18 922.14 ± 0.02 Da (calculated masses of RS20, calmodulin, and the calmodulin−RS20 complex are 2294.65, 16 627.37, and 18 922.02 Da, respectively). The observation of the calmodulin−RS20 complex constitutes direct and unambiguous evidence of the binding of apocalmodulin to the target site of MLCK.

Comparison of the spectrum of calmodulin alone with that of calmodulin in the presence of RS20 (Figures 1 and 3, respectively) indicates that the charge-state distribution patterns and relative intensities of the signals were altered upon addition of RS20. The envelope of the lower charge-states of the RS20-bound calmodulin was shifted relative to that of free calmodulin and was centered at the 9^+ charge-state for the RS20-bound calmodulin, cf. 8^+ for calmodulin alone. At the 1.5:1 concentration ratio, RS20 binding was observed at the lower charge-states of calmodulin only, i.e., there was no observable binding to unfolded conformations (see, however, discussion below).

The signals corresponding to dimeric calmodulin species disappeared upon addition of RS20 (Figure 3). This observation is interpreted in terms of changes in the dynamics of equilibria in solution brought about by RS20. The K_d for dimerization from ultracentrifugation was about 10^{-4} M in the absence of RS20 and of calcium, cf. the *K*d of 10^{-6}–10^{-7} M for peptide binding (*29*).

Experiments were carried out to test whether the calmodulin−RS20 complex could have dissociated to some extent in the gas phase, perhaps through loss of the peptide to give the folded calmodulin. Nozzle−skimmer and sustained off-resonance irradiation (SORI) experiments *(35)* showed that, upon excitation, the calmodulin−RS20 complex dissociated in the gas phase to the doubly charged peptide and calmodulin in the charge-state two less than that of the complex (i.e., $[C + P]^{9+}$, for example, gave $[C]^{7+}$ and $[P]^{2+}$). The absence of the doubly charged peptide from all of the spectra reported here confirmed that gas-phase dissociation of the calmodulin−RS20 complex was not significant under the conditions of these experiments. Similarly, SORI experiments on the calmodulin−RS20−Ca_4 showed that this complex, upon excitation, dissociated in the gas phase to the doubly charged peptide and calmodulin−Ca_4 (in the two-lower charge-state). Dissociation in the gas phase of the noncovalent complex calmodulin−RS20−Ca_4 through loss of the peptide can be firmly dismissed as a possibility under the conditions of the experiments reported, because there was no calmodulin−Ca_4 in the spectra (see below). By the same reasoning, a hypothesis that calmodulin−RS−Ca_2 was present but dissociated in the gas phase can be rejected, because there was no calmodulin−Ca_2 in the relevant spectra.

The effect of increasing the concentration of RS20 with respect to the calmodulin concentration was investigated. At lower concentration ratios (approximately equimolar RS20/calmodulin), RS20 was bound only at the lower charge-states of calmodulin (as shown in Figure 3). As the ratio of RS20 to calmodulin was increased, RS20 binding at higher charge-states was observed. At the same time, the higher charge-states became more pronounced relative to the lower charge-states until eventually the lower charge-states disappeared. At the highest concentration ratios, stoichiometries (RS20/calmodulin) of 2:1, 3:1, and even 4:1 were observed. The conclusion drawn is that the calmodulin structure had unfolded, to bind more than one peptide.

Calmodulin−RS20−Calcium Interaction. Panels a and b of Figure 4 show the ESI−FTICR mass spectra obtained for a solution of calmodulin in ammonium acetate buffer containing 0.01 mM calcium chloride in the absence and in the presence of RS20, respectively. The molar ratios of calmodulin to RS20 to $CaCl_2$ were 1:1.5:0.4.

The presence of calcium at this concentration (in the absence of RS20) did not greatly change the ESI mass spectrum (compare Figure 4a with Figure 1). Although there was a slight difference in the relative intensities of the 9^+ and 7^+ charge-states, the strongest peak remained that due to the 8^+ charge-state. Calcium binding was observed with one, two, three, and four calciums being evident at each of the charge-states. The relative intensities of these calcium-bound species varied slightly with charge-state. The presence of a calcium within a species was always associated with the loss of two hydrogens. In Figure 4, $[C + 1Ca]^{8+}$ represents [calmodulin + Ca − 2H + 8H]$^{8+}$, for example, and $[C + 4Ca]^{8+}$ represents [calmodulin + 4Ca − 8H + 8H]$^{8+}$.

In the presence of RS20, the most intense peak was due to the calmodulin−RS20−Ca_4 complex in the 8^+ charge-state (see Figure 4b, RS20 is denoted as P). There was slight evidence of calmodulin−RS20 complexes with one, two, or three calciums bound within the 9^+ charge-state, but none for either 7^+ or 8^+. There were strong peaks due to the protein alone and to the calmodulin−RS20 complex free of calcium. There was, however, no evidence, at these relative concentrations, of calmodulin bound to calcium alone (*without* RS20 also bound). There was no evidence of a calmodulin−RS20−Ca_2 complex. The near-complete absence of calmodulin−RS20 complexes with two calciums bound and the dominance of the calmodulin−RS20−Ca_4 complex is consistent with the idea of two globular domains functioning cooperatively (*43, 44*).

In the combined presence of RS20 and calcium, the unfolded conformations represented by the high charge-states were greatly diminished in their abundance. Calcium alone, at this concentration, did not significantly affect the abundance of unfolded conformations.

The ESI−FTICR mass spectrum of calmodulin, RS20, and calcium chloride when the salt concentration was raised to 100 μM is shown in Figure 5 (molar ratios of calmodulin to RS20 to calcium of 1:1.5:4). Higher charge-state species (i.e., unfolded calmodulin) disappeared at this increased concentration of calcium. The most abundant species remained the calmodulin−RS20−Ca_4 complex. Peaks corresponding to the unsaturated species $[C + 1Ca]^{8+}$, $[C + 2Ca]^{8+}$, $[C + 3Ca]^{8+}$, and $[C + RS20 + 1Ca]^{8+}$ were observed. At this high concentration, there was evidence of calmodulin−RS20 complexes binding more than four calciums (possibly as many as nine) *(16−18)*.

Calmodulin−RS20−Magnesium Interaction. The ESI−FTICR mass spectrum of calmodulin in the presence of RS20 and 0.3 mM magnesium chloride is shown in Figure 6 (calmodulin/RS20/$MgCl_2$ molar ratios were 1:1.5:12). The concentration of magnesium for this spectrum was 3 times higher than that of calcium in Figure 5. Comparison of Figures 5 and 6 shows that, as expected, calmodulin−RS20's affinity for magnesium was lower than its affinity for

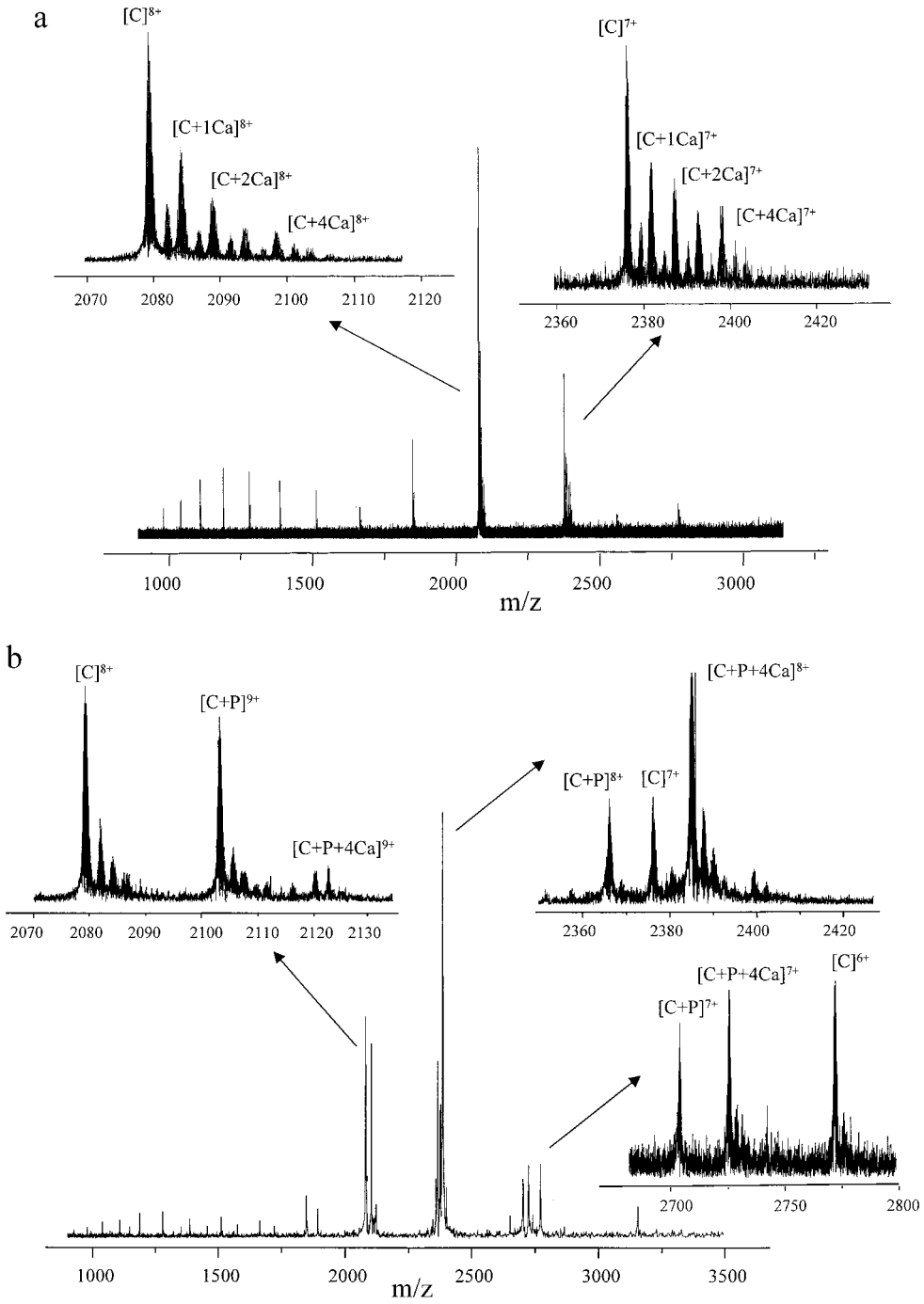

FIGURE 4: ESI−FTICR mass spectra of calmodulin in ammonium acetate buffer, 5 mM, pH 5.9, containing 0.01 mM $CaCl_2$ in (a) the absence of RS20, and (b) the presence of RS20 at a concentration ratio of 1:1.5. Insets (in panel a) show the expansion of the 8^+ and 7^+ charge-states. P (in panel b) represents RS20.

calcium. Considering Figure 6, the calmodulin−RS20 affinity for magnesium was not greater than that of calmodulin alone for magnesium (data not shown). The binding of the peptide was not significantly enhanced by the presence of magnesium. There was no preference for a 1:1:4 calmodulin/RS20/magnesium complex; a distribution of calmodulin−magnesium complexes was observed *without* any associated RS20.

CONCLUSION

The apocalmodulin−RS20 complex has recently been proposed to be the third physiologically relevant complex *(29)*, following the well-recognized calmodulin−RS20−Ca_4 complex as the first and a putative calmodulin−RS20−Ca_2 complex as a possible second. Our results indicate clearly that there was no RS20−CaM complex with two calcium ions bound under the conditions of our experiments. Our results are consistent with the conclusion of Tsvetkov et al. *(29)* that in the absence of calcium the RS20 peptide binds to the C-terminal lobe of calmodulin.

Our conclusions from mass spectrometry shed light on the question of how calmodulin recognizes its substrates. Wintrode and Privalov *(45)* have presented a view of the binding process in which the first step is either the occupation of all four calcium-binding sites or the occupation of the two calcium-binding sites at calmodulin's C-terminal domain. The second step is bridging by the peptide between the

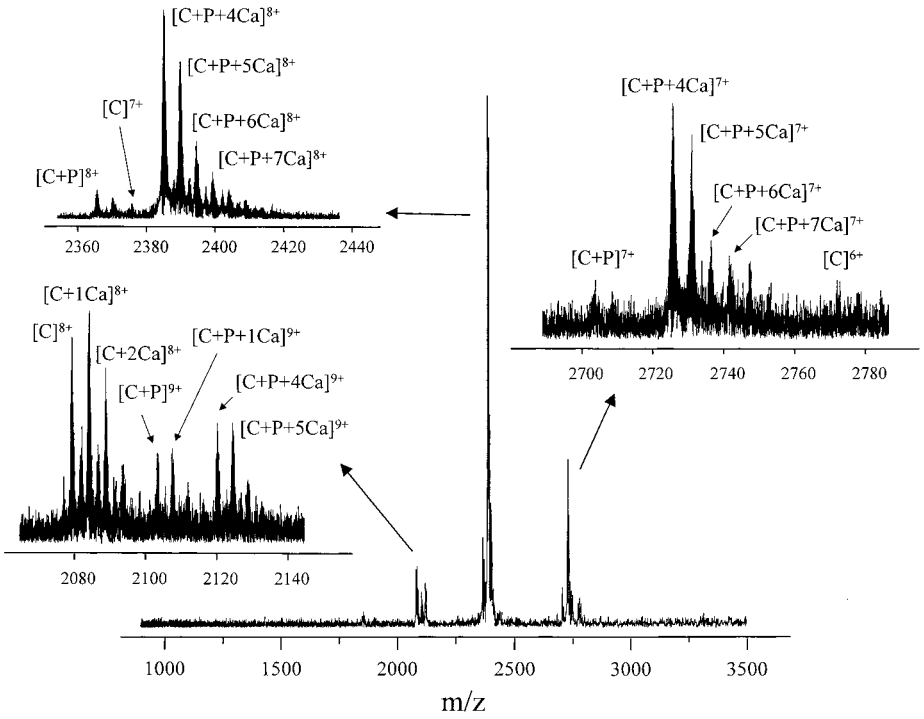

FIGURE 5: ESI−FTICR mass spectrum of calmodulin with RS20 peptide (concentration ratio 1:1.5) in ammonium acetate buffer, 5 mM, pH 5.9, containing 0.1 mM $CaCl_2$. Insets show the expansion of the lower charge-states. P represents RS20.

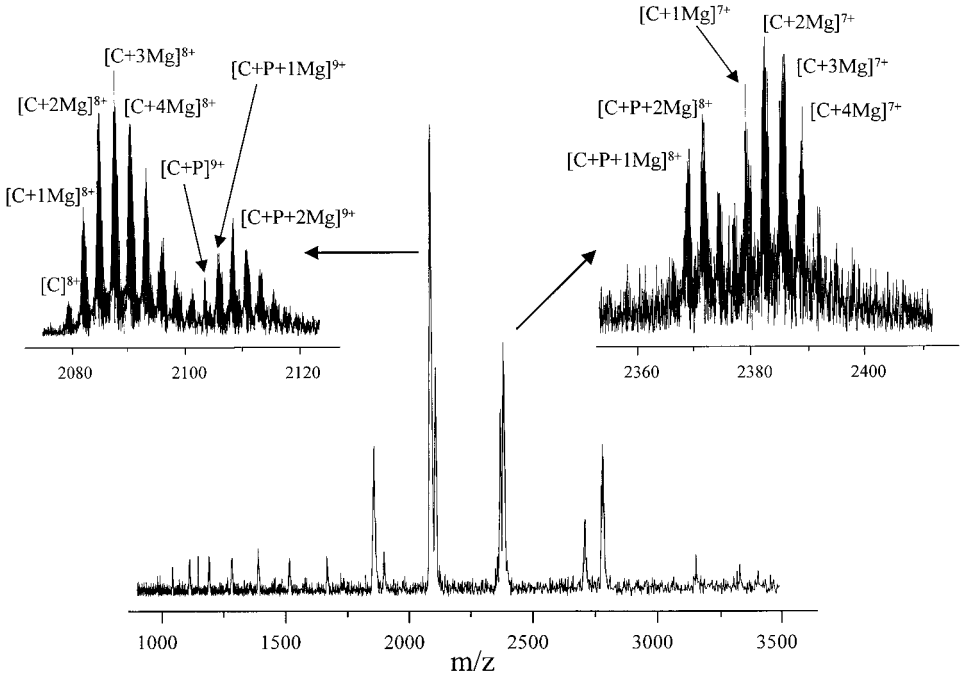

FIGURE 6: ESI−FTICR mass spectrum of calmodulin with RS20 peptide (concentration ratio 1:1.5) in ammonium acetate buffer, 5 mM, pH 5.9, containing 0.3 mM $MgCl_2$. Insets show the expansion of the lower charge-states. P represents RS20.

N-terminal and C-terminal domains of calmodulin when all four calcium-binding sites are full. If only the two C-terminal sites are occupied the peptide binds to the C-terminal domain only. The picture emerging from the mass spectrometric results would differ in the sequence of events, putting a different complexion on the question of substrate recognition *(46)*. The first step would be calmodulin binding with the peptide to form the complex apocalmodulin−RS20. The binding would initially occur between the C-terminal domain of calmodulin and the peptide, so that the peptide did not bridge the protein's termini *(29)*. Closing the bridge would be a subsequent step. Calcium would occupy the four calcium-binding sites, conferring additional stability and driving the equilibria toward calmodulin−RS20−Ca_4.

ACKNOWLEDGMENT

The authors thank Dr. J. Haiech for stimulating discussions, Dr. R. Toci and Dr. M. Cigna for their invaluable help in producing and purifying calmodulin, Dr. B. Calas for synthesis of RS20, and Professor C. Briand for providing the facilities to carry out the calorimetry experiments. The authors acknowledge Dr. X. Feng for technical support and

collaboration. The assistance and collaboration of Bruker Daltonics is gratefully acknowledged.

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BI000139M