Analysis of the Ion Binding Sites of Calmodulin by Electrospray Ionization Mass Spectrometry†

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ABSTRACT: The binding of Ca^{2+} and Mg^{2+} to four calmodulins (SynCaM 1, SynCaM 8, SynCaM 12A, and SynCaM 18A) has been studied by ESI-MS. The mass spectra were recorded by dissolving the apoproteins in methanol/water (20/80, v/v) containing 1 mM $CaCl_2$ or 1 mM $MgCl_2$ and the pH adjusted to 6.0 with ammonia. The carrier solvent was methanol/water (20/80, v/v). In the case of Ca^{2+} complexation, ESI-MS reveals the presence of three kinds of sites: the first of high affinity corresponding to those determined using flow and equilibrium dialysis techniques and two others with lower affinities. These results clearly confirm the conclusion of Milos et al. [Milos, M., Comte, M., Schaer, J. J., & Cox, J. A. (1989) *J. Inorg. Biochem. 36,* 11−25] that there should exist between four and six auxiliary sites for Ca^{2+}. Concerning the complexation of magnesium, the four proteins are able to bind two Mg^{2+} almost certainly on auxiliary cationic sites.

Calmodulin (CaM)[1] is a small (148 residues), acidic, and highly conserved calcium binding protein found in all eucaryotic cells (Kretsinger, 1980; Van Eldick et al., 1982; Cohen & Klee, 1988). Its primary function is the modulation of the activity of a large number of enzymes (cyclic nucleotide phosphodiesterase, adenyl cyclase, Ca^{2+}−Mg^{2+} ATPase, calcineurin, Ca^{2+}−calmodulin-dependent kinases, and myosin light chain kinases) in response to changes in calcium concentration (Kretsinger, 1980; Van Eldick et al., 1982; Cox et al., 1984; Forsén et al., 1986; Lukas et al., 1988; Cohen & Klee, 1988). The regulation occurs through strong interactions between specific domains of the Ca^{2+}−CaM complex and specific domains of the regulated enzymes (Clore et al., 1993).

Biological processes involving CaM are of primary importance and include muscle contraction, cellular metabolism, cellular motility, division and growth, membrane permeability, and ion transport (Payne & Rudnick, 1989; Berridge, 1990; Pietrobon et al., 1990; Heizman & Hunziker, 1991). As a result, there have been numerous studies of the calcium binding mechanism at the molecular level (Kilhoffer et al., 1983, 1988, 1992; Mills & Jonhson, 1985; Milos et al., 1986, 1989; Porumb, 1994). These studies have established that CaM can bind four Ca^{2+} between pH 6 and 8. Application of the Klotz−Adair binding model (Haiech et al., 1981; Klotz, 1985; Kilhoffer et al., 1988; Porumb, 1994) yields four macroscopic dissociation constants: $K_1 = 2 \times 10^{-5}$, $K_2 = 9 \times 10^{-5}$, $K_3 = 2.9 \times 10^{-4}$, and $K_4 = 1 \times 10^{-5}$ M^{-1}.

The three-dimensional structure of Ca^{2+}−CaM was determined in 1985 (Babu et al., 1985, 1988; Kretsinger et al., 1986; Strynadka & James, 1991; Taylor et al., 1991). It is a dumbbell shaped molecule with two roughly globular domains linked by a long solvent-exposed helix. Each globular domain contains two Ca^{2+}-binding sites, each consisting of a helix−loop−helix motif (Falke et al., 1994). The mechanism of calcium binding is complex. The most satisfactory model (Haiech et al., 1981; Kilhoffer et al., 1992) assumes a sequential binding: III → IV → I → II (for numbering of the sites, see Figure 1).

Indirect evidence suggested (Mills & Jonhson, 1985; Milos et al., 1986, 1989) that CaM contains auxiliary sites in addition to the four Ca^{2+} essential sites necessary for enzyme binding. According to Milos et al. (1986, 1989), between four and six such auxiliary sites should exist, with dissociation constants in the millimolar range for Ca^{2+}. Some cations such as Ca^{2+} and Sr^{2+} would bind selectively to the principal sites; other such as Zn^{2+}, Mn^{2+}, Cu^{2+}, and Hg^{2+} would bind specifically to the auxiliary sites; while a third class such as La^{3+}, Tb^{3+}, Pb^{2+}, and Cd^{2+} could bind to both types (Milos et al., 1989).

Due to their low affinity, the auxiliary sites are difficult to reveal. It is of primary importance to demonstrate clearly their existence and to determine their number, knowledge of which is fundamental for an appropriate modeling of experimental data (Klotz, 1985; Porumb, 1994).

We have attempted to determine the correct stoichiometry of the Ca^{2+}−CaM and Mg^{2+}−CaM complexes by using ESI-MS. ESI is a gentle ionization technique allowing the transfer of ions from solution to gas phase for mass spectrometric analysis (Kebarle & Tang, 1993). Numerous recent reports suggest that it may be useful for probing molecular noncovalent interactions that occur in the solution phase [see, for example, Jaquinod et al. (1993), Smith and Light-Wahl (1993), and Loo et al. (1994)]. Consequently,

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[1] Abbreviations: CaM, calmodulin; SynCaM, synthetic calmodulin; ESI-MS, electrospray ionization mass spectrometry.

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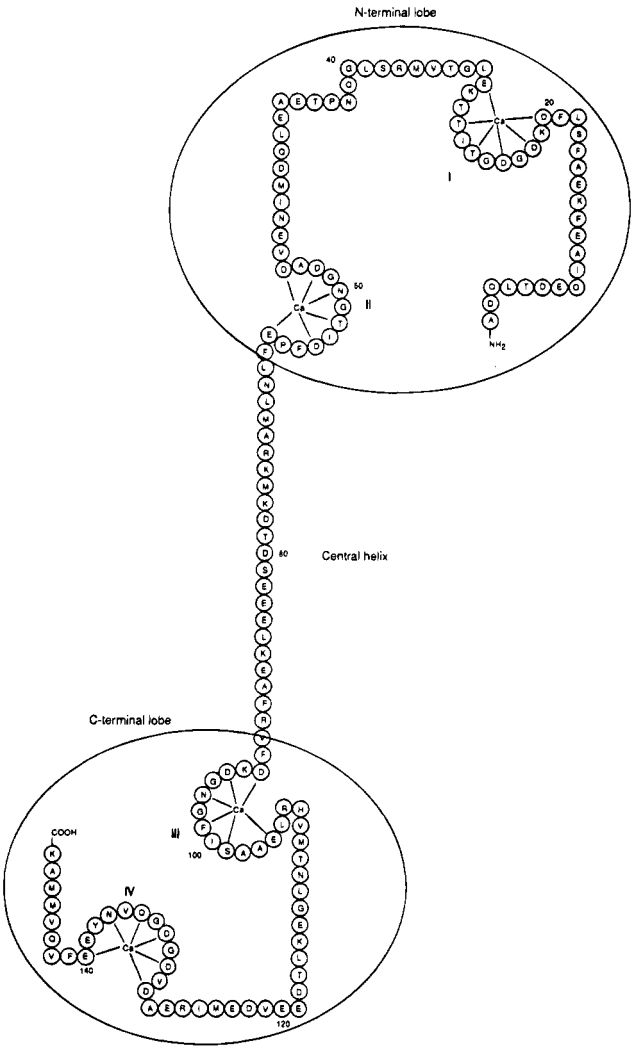

FIGURE 1: Sequence of SynCaM 1. In SynCaM 8, -^{82}EE^{84}E- is replaced by -^{82}KK^{84}K- and in SynCaM 12A -^{118}EE^{120}E- by -^{118}KK^{120}K-. SynCaM 18A combines these two mutations.

it appears possible to deduce the number of metal ions present in the complex M^{n+}−CaM from the mass difference between holoprotein and apoprotein. However, the problem is not trivial, as it is necessary to maintain the specific noncovalent interactions between the protein and the metal during the generation and the transfer of ions from liquid to gas phase (Smith & Light-Wahl, 1993). For this purpose, the compound must be injected in a solution which preserves the form with metal bound until spray volatilization. Obviously, the composition (presence or absence of an organic solvent) and the pH of this solution are of prime importance.

In this work, we have studied the interaction between synthetic calmodulin SynCaM 1 and three mutants (SynCaM 8, SynCaM 12A, and SynCaM 18A, see Figure 1) with two divalent cations (Ca^{2+} and Mg^{2+}) of physiological importance.

Using positive ion ESI-MS and conditions which allow the specific interactions between the metal and the proteins to be preserved, we have measured the total number of cations bound to the four SynCaMs.

MATERIALS AND METHODS

General Indications. Solutions were made with MilliQ water treated before use with Chelex 100 to remove traces of Ca^{2+}. All the plastic utensils used in this work (beakers, columns, tips, etc.) were washed with 1 M HCl and extensively rinsed with decalcified water. Methanol and acetonitrile (HPLC grade) were purchased from SDS (Peypin, France).

Production of SynCaM 1, SynCaM 8, SynCaM 12A, and SynCaM 18A. SynCaM 1, SynCaM 8, SynCaM 12A, and SynCaM 18A were obtained and purified as previously described (Roberts et al., 1985; Craig et al., 1987). The purity of proteins was assessed by SDS gel electrophoresis, capillary electrophoresis, high-pressure liquid chromatography, and ESI-MS. The amino acid analyses are in good agreement with the proposed sequences.

Electrospray Ionization Mass Spectra. ESI-MS spectra were acquired on a VG Trio-2000 mass spectrometer (Fisons/VG, Manchester, U.K.) with an atmospheric pressure electrospray ion source and an interface employing a 75 μm i.d. fused silica capillary inlet. The masses were analyzed through a quadrupole with a maximum mass range of 3000. The mass spectrometer was scanned from *m/z* 600 to 1900 in 10 s. Fifteen scans (10 s each) were averaged to obtain each final spectrum. The electrospray carrier solvents used were either acetonitrile/water (50/50, v/v) or methanol/water (50/50 and 20/80, v/v). The flow rate was 2 μL/min. Sample injection was carried out by means of a Rheodyne 8125 injector valve equipped with a 10 μL sample loop. Desolvation of the analyte ions was achieved in part by controlled heating of the capillary and in part by collision activation brought about by an electrostatic field in the intermediate pressure region between the capillary exit and a coaxial skimmer (sampling cone voltage). The capillary tip voltage and the counterelectrode voltage were optimized at 3 and 0.7 kV, respectively. The temperature of the capillary was set at 64 °C for the metal-free proteins and lowered to 35 °C for the complexes. The mass scale had been calibrated before and then intermittently during the sequence of analyses using horse heart myoglobin (MW 16951.5, Sigma Chemical Co., U.K.). Instrument control, data acquisition, and some preliminary postrun processing used the VG LabBase software supplied with the instrument. Presentation of the raw *m/z* data onto a true molecular weight scale employed the transformation software of the MassLynx package supplied with the VG Trio-2000. Precise molecular weight were determined using the maximum-entropy software (MaxEnt) incorporated into the VG MassLynx software package. Before the injection of proteins with metal bound, the capillary was washed with formic acid and then rinsed for 12 h with the carrier solvent (water−acetonitrile or water−methanol) in order to remove traces of acid. All masses given in this paper are average molecular masses.

As ESI-MS can generate artifacts, we considered that measures concerning a complex are reliable if the spectra of five independent samples of the same complex were nearly identical.

Sample Preparation. Proteins (2 mg, about 10^{-7} mol) were dissolved in 1 mL of water, and then 33 μL of a solution of trichloroacetic acid/water (1% w/v) was added. The suspension was centrifuged, and the precipitate was collected and dissolved in 33 μL of 1 M Tris (pH 9.5) and 967 μL of water. Thirty-three microliters of trichloroacetic acid/water (1% w/v) was added again and the mixture centrifuged. The precipitate was suspended in water and 33% ammonia added until complete dissolution (about 3 μL). This solution was

chromatographed on a PD10 column (Pharmacia, Uppsala, Sweden). The fractions containing the protein were pooled (about 1 mL of resulting solution) and freeze-dried. The solid was dissolved in methanol/water (20/80, v/v), a solution of 1 M $CaCl_2$ (or 1 M $MgCl_2$) was added until a molarity close to 1 mM, and the pH was brought to 6 with 1 M ammonia. This solution was centrifuged for 10 min at 5000 rpm to prevent clogging of the capillary by particulates, and 10 μL (100 pmol/μL) of the supernatant was injected to acquire ESI mass spectra.

RESULTS AND DISCUSSION

SynCaM 1 is a synthetic protein hybrid of mammalian and plant calmodulin able to activate all the Ca^{2+}−calmodulin-dependent enzymes. SynCaM 8, 12A, and 18A have been designed (Roberts et al., 1985; Craig et al., 1987) to examine the incidence of electrostatic potential on the coupling between the four Ca^{2+} binding sites. The modifications carried out in regard to SynCaM 1 do not affect the Ca^{2+} binding loops but their flanking parts only. In SynCaM 8 the sequence -^{82}EE^{84}E- is replaced by -^{82}KK^{84}K- and in SynCaM 12A -^{118}EE^{120}D- by -^{118}KK^{120}K- (Figure 1). SynCaM 18A combines these two mutations (Figure 1).

The molecular masses of SynCaM 1, 8, 12A, and 18A, determined by ESI-MS in acetonitrile/water/formic acid (49.5/49.5/1, v/v/v) were 16 628.4 ± 3.8, 16 626.1 ± 4.2, 16 636.8 ± 3.1, and 16 635.8 ± 5.4 Da, respectively. They are in good agreement with the values calculated from the sequences (SynCaM 1, 16 627.4; SynCaM 8, 16 624.5; SynCaM 12A, 16 638.6; and SynCaM 18A, 16 635.7).

Interaction between SynCaMs and Ca^{2+}. ESI-MS analyses can be performed in positive or in negative mode. In positive mode, traces of acid (formic, acetic or trifluoroacetic) were added to favor multiple protonation of the protein. In negative mode, addition of bases such as ammonia promotes multiple deprotonation. The choice between these two modes depends on the nature of the molecule analyzed (protein, nucleic acid, or polysaccharide). Usually, the instrument polarity is chosen of the same sign that the net charge of the species studied. For example, bovine calmodulin, which contains 39 acidic residues and 15 basic (isoelectric point close to 4), seems to require the use of negative ESI-MS. This polarity should lead to an optimal sensitivity owing to the large excess of COO^- in regard to NH_3^+, within the pH range 6−8 where the protein exhibits the higher affinity for Ca^{2+}. Recently, Hu et al. (1994) have determined the Ca^{2+} binding stoichiometry for some calcium binding proteins (bovine calmodulin, α-lactalbumin, and rabbit parvalbumin) using negative ESI-MS for bovine calmodulin and rabbit parvalbumin and positive ESI-MS for α-lactalbumin. According to these authors, bovine calmodulin would bind four Ca^{2+}, and no auxiliary sites were detected (Hu et al., 1994). This result did not agree with the works of Milos et al. (1986, 1989), for whom it is necessary to assume the presence of extra sites to interpret the thermodynamic features of Ca^{2+} binding to CaM.

Consequently, we have reconsidered the ESI-MS studies of Hu et al. (1994). For this purpose, we have registered the mass spectrum in negative mode of SynCaM 1, in 1 mM $CaCl_2$ (pH 8.5) without methanol; it reveals the presence of a largely major species containing four Ca^{2+}. The absence of auxiliary sites could be related to the use of negative mode. Protonation and deprotonation of proteins in solution can modify their three-dimensional structure and can influence the noncovalent binding of the ligand. It is possible that the auxiliary sites in calmodulin are unable to bind Ca^{2+} at basic pH. Therefore, we have attempted to study SynCaMs in ESI-MS by using a positive polarity for the spectrometer.

The isoelectric point of CaMs ranges from 3.5 to 4, but it must be realized that this parameter has been measured *under denaturing conditions* (Bjellqvist et al., 1993), and consequently the ionization state of native CaMs is unknown. It is well established that the pK_a values of ionizable groups depend on many environmental and electrostatic effects (Creighton, 1993). The variety of environments in folded proteins can produce very unusual ionization properties. The pK_a values of residues of one type can vary widely within a single protein (often a range of 3−4 pH units). In CaMs, it is possible that several carboxylic groups are not easily ionizable owing to environmental effects occurring in the compact structure of the N and C terminal lobes. This assumption allows the appearance of the negative ESI-MS of bovine calmodulin (Hu et al., 1994) to be explained: only 16 negative ions are detected. Among the 39 COOH groups present, 16 are ionized and 8 strongly linked to four Ca^{2+}; the 15 remaining groups must be buried within the protein. In CaMs between pH 6 and 8 the ratio COO^-/NH_3^+ should be close to 1. Consequently, it appears possible to study the stoichiometry of Ca^{2+} binding to these proteins by using positive ESI-MS. Hu et al. (1994) argued that positive detection has two major disadvantages: a low sensibility and a cationization due to the presence of Na^+. Here, we have demonstrated that the first of these disadvantages is unfounded, since the positive ESI-MS of calmodulins can be easily acquired despite their "apparent" low isoelectric point. Concerning the cationization by Na^+, it is necessary to remove traces of this ionic contaminant. Na^+ does not compete with Ca^{2+}, since it is bound to different positions. Consequently, if special care is not taken, the Ca^{2+}−CaM complexes may contain variable amounts of Na^+, so that the interpretation of mass spectra becomes very difficult. The absence of Na^+ requires (i) a careful desalting of protein samples, (ii) the use of highly purified water and organic solvents, (iii) the washing before injection of the fused silica capillary with formic acid followed by an overnight washing with the carrier solvent, and (iv) the exclusive use of plastic rather than glass vessels. With these stringent conditions, calmodulins are sodium free.

In first attempts, SynCaM 1 was decalcified by treatment with trichloroacetic acid and chromatographed in water on PD10 column. The fractions containing the protein (about 1 mL) were pooled, freeze-dried, and dissolved in acetonitrile/water (50/50, v/v) or methanol/water (50/50, v/v). SynCaM 1 was then recalcified by adding 1 M $CaCl_2$ up to a molarity close to 1 mM. The pH was adjusted to 6 with 1 M ammonia, and 10 μL of the solution was injected with acetonitrile/water or methanol/water (50/50, v/v) as carrier solvent. Using this procedure precipitation can occasionally occur giving capillary leakage and detection of different molecular species. First, decalcified protein appears, then successively several metallic adducts with different Ca^{2+} contents, and finally decalcified protein again. Such a behavior may correspond to total and partial denaturation of SynCaM 1 due to gradual mixing in the capillary between the carrier solvent and the aqueous solution of the protein.

Table 1: Numbers of Ca^{2+} Bound to Species Corresponding to Peaks 1, 2, and 3 in SynCaMs[a]

	peak 1	peak 2	peak 3
SynCaM 1	$3.9 \pm 0.3 \leq n \leq 4.7 \pm 0.3$ (4)	$5.9 \pm 0.4 \leq n \leq 6.7 \pm 0.4$	$9.1 \pm 0.2 \leq n \leq 9.7 \pm 0.2$
SynCaM 8	$4.1 \pm 0.4 \leq n \leq 4.8 \pm 0.4$ (4)	$6.1 \pm 0.4 \leq n \leq 6.8 \pm 0.4$	$8.9 \pm 0.5 \leq n \leq 9.3 \pm 0.5$
SynCaM 12A	$3.1 \pm 0.3 \leq n \leq 3.6 \pm 0.3$ (3)	$6.1 \pm 0.5 \leq n \leq 6.8 \pm 0.5$	$9.1 \pm 0.4 \leq n \leq 9.6 \pm 0.4$
SynCaM 18A	$1.3 \pm 0.3 \leq n \leq 2 \pm 0.3$ (2)	$6.2 \pm 0.5 \leq n \leq 6.7 \pm 0.5$	$8.7 \pm 0.5 \leq n \leq 9 \pm 0.5$

[a] In parentheses are reported the number of Ca^{2+} bound to SynCaMs determined by dialysis techniques.

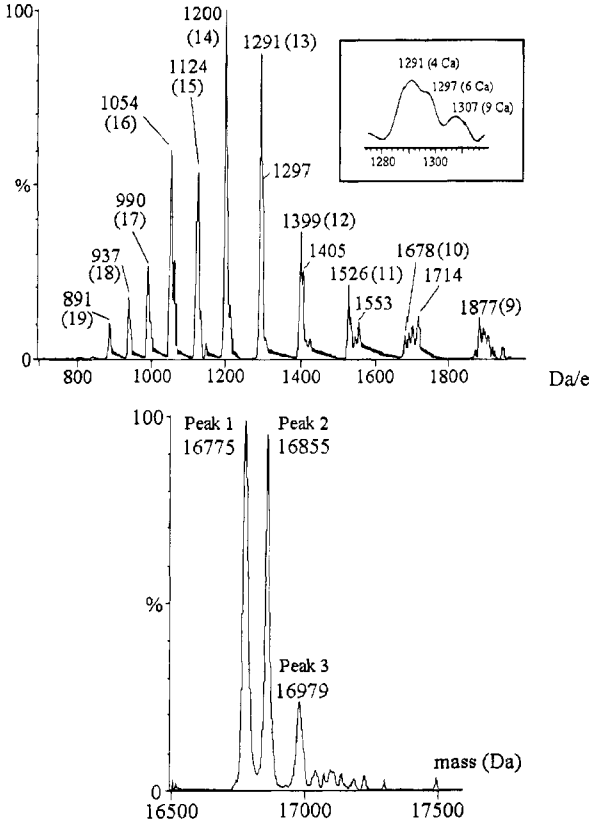

FIGURE 2: Complexes of SynCaM 1 and Ca^{2+}. (Upper pannel) Positive ion ESI-MS (raw data). (Lower panel) Mass spectrum produced by MaxEnt processing of the raw electrospray data. (Inset) Enlargment of the raw signal corresponding to 13 charges showing the presence of several molecular species according to the number of Ca^{2+} bound.

To overcome this problem, we have looked for a nondenaturing carrier solvent. After numerous attempts, we have chosen 20% methanol/water (v/v). In these conditions (CH_3OH/H_2O, 1 mM $CaCl_2$, pH 6), it is possible to obtain stable and reproducible mass spectra. However, the presence of an important level of fragmentation was observed, which was eliminated by using a lower sampling cone voltage value (45 V).

Usually, in ESI-MS, the source temperature is kept close to 65 °C. According to the recommendation of the manufacturer (VG TRIO 2000, Users Guide, Fison Instrument), if more than 50% water is present in the mobile phase a higher temperature should be used. However, in this work where 20% methanol in water was used, the best results were obtained with a source temperature of 35 °C.

The ESI-MS (positive mode) of SynCaM 1$-Ca^{2+}$ reported in Figure 2 reveals the presence of three species. The raw data were analyzed by MaxEnt at high resolution (1 Da/channel) over a narrower mass range in order to provide optimal resolution of the mixture (Evershed et al., 1993). As expected, three peaks, 1, 2, and 3, are detected. If we compare the mass determined for the more intense one, peak 1 (16 775 Da), with the molecular mass of the decalcified protein (16 627 Da), the difference (148 Da) could correspond to the fixation of 3.7 Ca^{2+}, close to the value determined by dialysis techniques (Kilhoffer et al., 1988; Haiech et al., 1991). However, it may be noted that MaxEnt gives a mass M_A corresponding to $M_A = M_0 + n40 - 2n - x$ (eq 1), where n is the number of Ca^{2+} bound to the protein, M_0 the calculated molecular weight of the decalcified protein, and x the number of deprotonated carboxylic groups. Indeed, when one Ca^{2+} is incorporated into CaMs, two protons by Ca^{2+} are displaced to maintain the charge balance. In addition, x protons are lost due to the ionization of COOH at the pH used. In these conditions, the number of Ca^{2+} incorporated n [$n = (M_A - M_0 + x)/38$] is dependent on the extent of deprotonation x. For reasons discussed above, this parameter cannot be known. In order to estimate n, we have considered two limit states for each species corresponding to peaks 1, 2, and 3. In the first one we have assumed a complete deprotonation for the COOH of the protein, $2n + x$ in eq 1 remains equal to the total number X of carboxylic groups present in the protein [$n = (M_A - M_0 - X)/40$]. In the second state, we have assumed that the COOH are not ionized (except those implicated in the calcium binding), so that x becomes equal to 0 [$n = (M_A - M_0)/38$]. Obviously, the true values of n must be between these two limits. The results are reported in Table 1.

Considering the results of flow dialysis experiment (Kilhoffer et al., 1988; Haiech et al., 1991) and assuming the fact that the difference between the masses of decalcified protein and of SynCaM 1-$(Ca^{2+})_n$ is obligatory equal to an integer number of Ca^{2+}, we can say that the lower limit given in Table 1 is certainly the right value for SynCaM 1. Therefore the complexes corresponding to peaks 1, 2, and 3 (Figure 2) are SynCaM 1$-(Ca^{2+})_4$, SynCaM 1$-(Ca^{2+})_6$, and SynCaM 1$-(Ca^{2+})_9$, respectively (Table 1). The first one may be related to the binding of Ca^{2+} on the four high affinity sites (principal sites) and the second and the third ones to the binding at auxiliary sites.

The maximum ionization observed for SynCaM 1 (+19, Figure 2) is higher than the number of the easily protonable side chain groups (+17: 9 Lys, 5 Arg, the terminal NH_2, and 1 His with two sites of protonation). The two additional charges may be due to the protonation of proline, asparagine, or glutamine as previously reported (Jaquinod et al., 1992; Moore et al., 1992). This indicates that the NH_2 groups of Lys, Arg, and His are easily accessible, in contrast to the COOH groups as observed in negative ESI-MS.

The positive ESI-MS of SynCaM 1 (Figure 2), SynCaM 8, 12A, and 18A (Figure 3) present differences in the ionization number and in the position of the most abundant ion. This can be related to differences in the three-dimensional structure of the four proteins which induce a more or less important ease of protonation. However, the

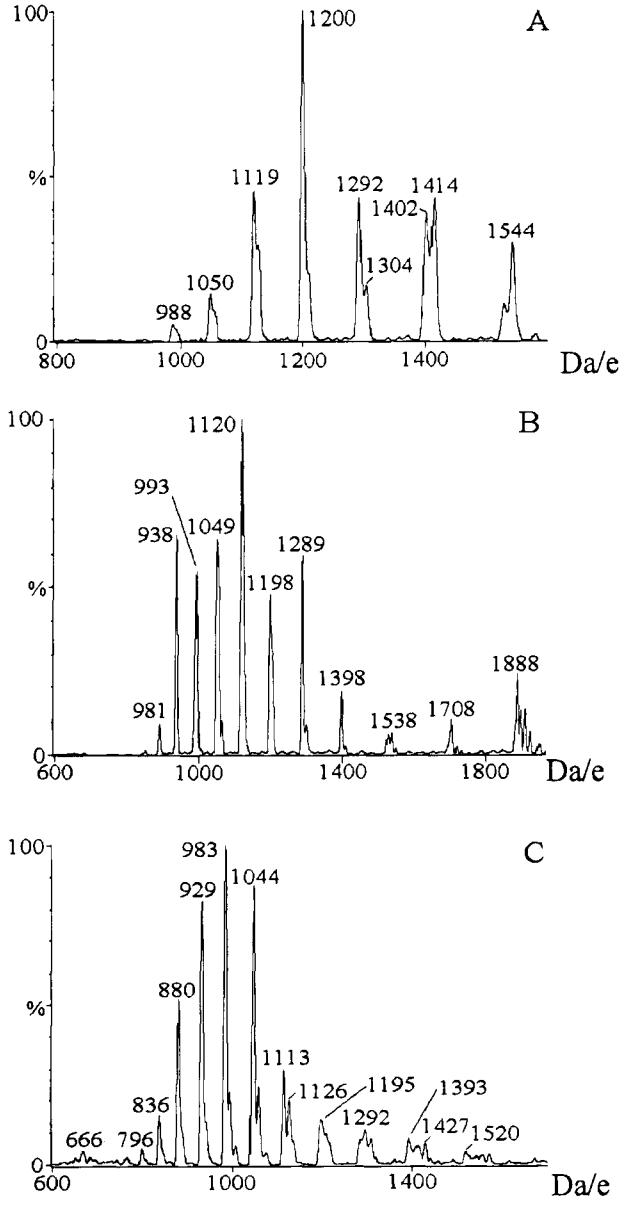

FIGURE 3: Complexes of SynCaMs and Ca^{2+}. Positive ion ESI-MS (raw data) of SynCaM 8 (A), SynCaM 12A (B), and SynCaM 18A (C).

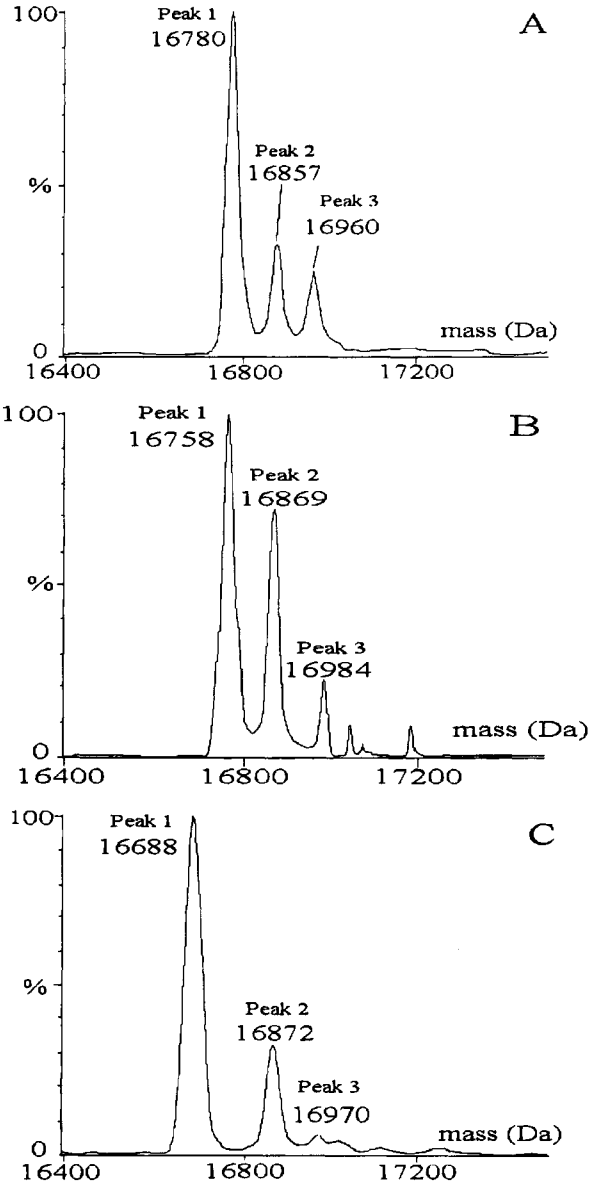

FIGURE 4: Complexes of SynCaMs and Ca^{2+}. Mass spectra produced by MaxEnt processing of the raw electrospray data: (A) SynCaM 8, (B) SynCaM 12A, and (C) SynCaM 18A.

transformation of raw data to masses by using MaxEnt for SynCaM 8, 12A, and 18A gives results (Figure 4) similar to those reported for SynCaM 1: three peaks 1, 2, and 3 are detected.

If we consider the lower limit given in Table 1 for SynCaM 8 and SynCaM 12A and the upper limit for SynCaM 18A, the number of Ca^{2+} present in the species corresponding to the most abundant peak 1 is in good agreement with that determined for the principal Ca^{2+} binding sites by using flow dialysis in Hepes 50 mM, pH 7.5 (Lafitte et al., unpublished data).

Complexes corresponding to peaks 2 and 3 bind about six and nine Ca^{2+}, respectively (Table 1). Consequently, two (for SynCaM 1 and 8), three (for SynCaM 12A), and four (for SynCaM 18A) identical auxiliary Ca^{2+} binding sites are present in class 2 (Figure 5). Owing to the mutations carried out in the sequences of SynCaM 12A and 18A, it must be noted that one and two of the principal sites over the four possible are respectively transformed into auxiliary sites in

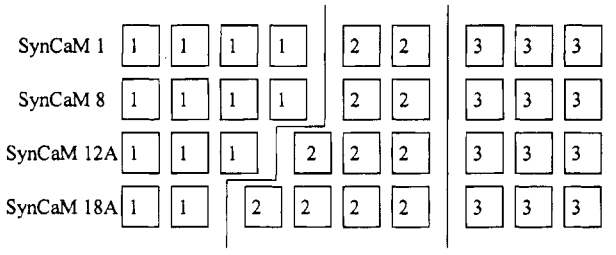

FIGURE 5: Schematic representation of Ca^{2+} binding sites in SynCaMs. Class 1, high affinity sites; class 2 and class 3, auxiliary calcium binding sites.

these two proteins. The ability of calcium binding persists, but the affinity is significantly reduced. We observe the addition of three additional Ca^{2+} located on three identical sites (class 3, Figure 5).

For SynCaM 1, 8, 12A, or 18A, we did not observe the presence of species: carrying zero Ca^{2+} (10^{-3} M Ca^{2+} is sufficient for saturating the principal sites) or having intermediate stoichiometries between those of the three

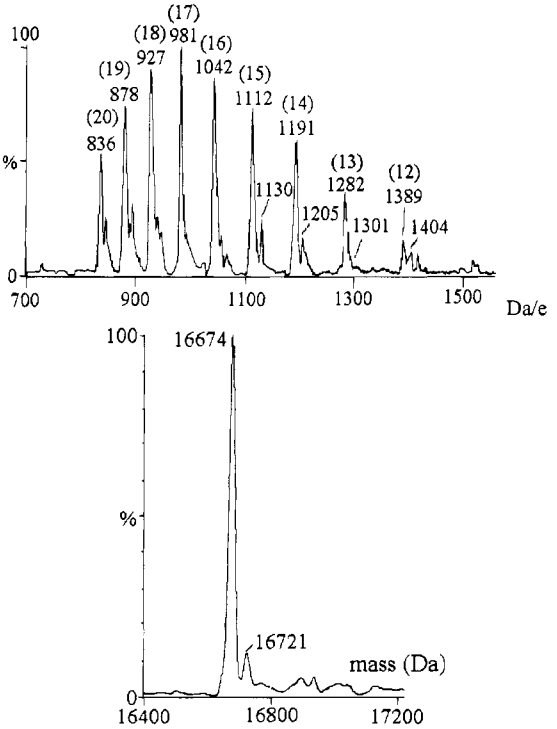

FIGURE 6: Complexes of SynCaM 1 and Mg^{2+}. (Upper panel) Positive ion ESI-MS (raw data). (Lower panel) Mass spectrum produced by MaxEnt processing of the raw electrospray data.

Table 2: Numbers of Mg^{2+} Bound to SynCaMs (See Figures 6 and 8)

SynCaM 1	$2.1 \pm 0.1 \leq n \leq 3.8 \pm 0.1$
SynCaM 8	$2.1 \pm 0.4 \leq n \leq 3.7 \pm 0.4$
SynCaM 12A	$2.3 \pm 0.4 \leq n \leq 3.9 \pm 0.4$
SynCaM 18A	$0 \leq n \leq 1.7 \pm 0.4$

complexes: SynCaMs$-(Ca^{2+})_n$ ($2 \leq n \leq 4$), SynCaM$-(Ca^{2+})_6$, and SynCaM$-(Ca^{2+})_9$.

Study of the Interaction between SynCaMs and Mg^{2+}. Complexes between Mg^{2+} and SynCaMs were prepared as described above, except that the decalcified proteins were treated with $MgCl_2$ instead of $CaCl_2$. ESI-MS spectra were recorded as previously reported for Ca^{2+} and SynCaMs. The raw mass spectrum concerning the positive ESI-MS of SynCaM 1$-Mg^{2+}$ is reported in Figure 6; the shape seems to be indicative of the presence of a major species. This is confirmed by MaxEnt analysis (Figure 6). The difference between the mass of the complex and that of the metal-free SynCaM 1 corresponds to the binding of 1.74 Mg^{2+} (the small peaks occurring at the side of the base peak correspond to a nonspecific binding since their positions vary according to the measurement). However, one must take into account the deprotonation of carboxylic groups as serious errors can be introduced especially with light ions such as Mg^{2+}. Using the method reported above, we have calculated the number of Mg^{2+} bound to SynCaM 1, considering a fully ionized and an unionized state for the COOH groups of the protein. The results are reported in Table 2. If we take in account the lower limit as for Ca^{2+} binding, SynCaM 1 appears able to bind 2 Mg^{2+}. The raw and the transformed (by MaxEnt) mass spectra for SynCaM 8, 12A, and 18A are reported in Figures 7 and 8; they are essentially the same as the SynCaM 1 spectrum (Figure 6). Like this protein, SynCaM 8 and 12A (considering the lower limit as for Ca^{2+} binding) and SynCaM 18A (upper limit) can bind 2 Mg^{2+} (Table 2).

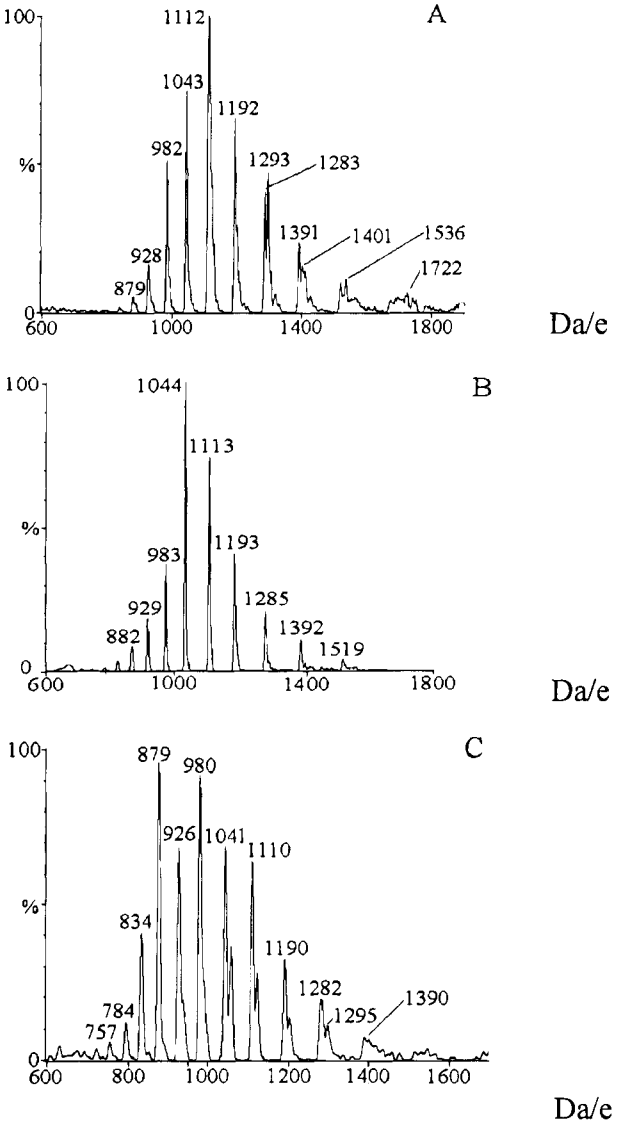

FIGURE 7: Complexes of SynCaMs and Mg^{2+}. Positive ESI-MS (raw data) of SynCaM 8 (A), SynCaM 12A (B), and SynCaM 18A (C).

Mg^{2+} binding to the different synthetic calmodulins is remarkably different from the Ca^{2+} binding complexes. Only one species may be detected in presence of 1 mM $MgCl_2$. This suggests, under our experimental conditions, an affinity higher than 10^{-4} M^{-1}. Tsai et al. (1987) have suggested that Mg^{2+} binds to calmodulin on the four helix−loop−helix motifs with an affinity in the 10^{-4} M^{-1} range for the two sites of the N-terminal lobe and a 10^{-3} M^{-1} affinity for the two other of the C-terminal lobe on the basis of Mg^{25} NMR experiments. Such an analysis has not taken into account the possibility for Mg^{2+} to bind only to auxiliary sites that interact with the helix−loop−helix motifs. On the other hand, on the basis of calorimetric studies, Milos et al. (1986, 1989) excluded the possibility for Mg^{2+} to compete directly with Ca^{2+} on the helix−loop−helix motifs. As the Mg^{2+} binding of the different SynCaMs is pretty similar, at the opposite of Ca^{2+} binding, this suggests that Mg^{2+} binds to auxiliary sites (the class 2 and the class 3 of the Ca^{2+} binding sites, Figure 5). This result is in agreement with the model of Milos et al. (1986, 1989).

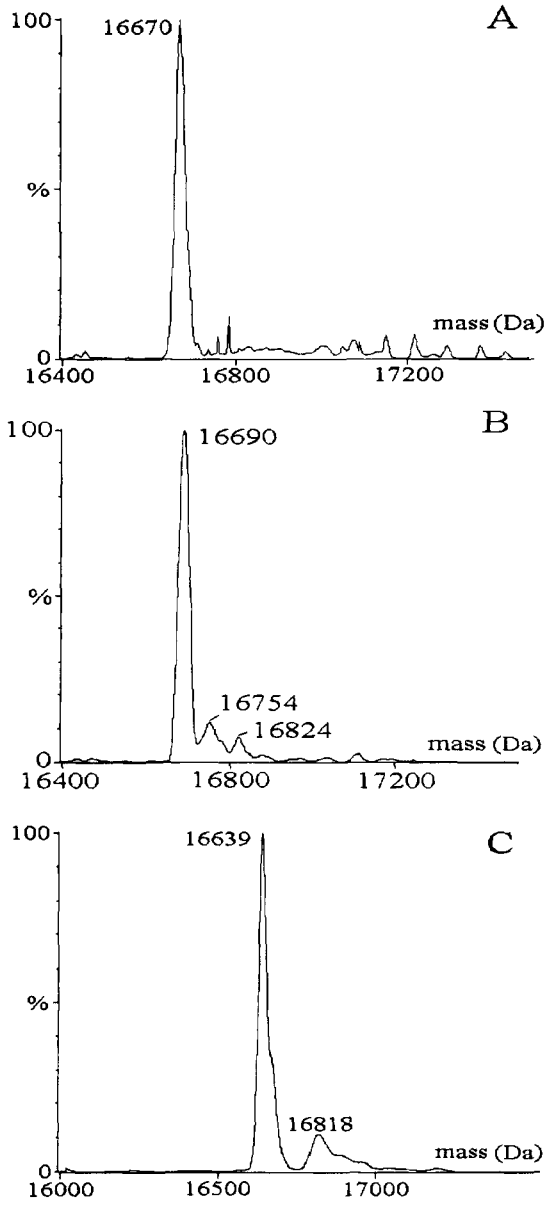

FIGURE 8: Complexes of SynCaMs and Mg^{2+}. Mass spectra produced by MaxEnt processing of the raw electrospray data: (A) SynCaM 8, (B) SynCaM 12A, and (C) SynCaM 18A.

CONCLUSION

This study of Ca^{2+} bound to SynCaMs by positive ESI-MS allows three species to be visualized: (i) a major species that corresponds to CaM-$(Ca^{2+})_n$ ($2 \leq n \leq 4$) (sites of class 1, Figure 5), (ii) one corresponding to CaM$-(Ca^{2+})_6$ (sites of class 2, Figure 5), and (iii) a minor one corresponding to CaM$-(Ca^{2+})_9$ (sites of class 3, Figure 5)

Due to the qualitative differences of the three species, we suggest that the CaM$-(Ca^{2+})_n$ complex corresponds to CaM with the n helix−loop−helix domains occupied and that CaM$-(Ca^{2+})_6$ and CaM$-(Ca^{2+})_9$ complexes suggest the existence of five auxiliary sites. These sites may be subdivided in two sets, a set with moderate affinity (two equivalent sites) and a set with a low affinity (three equivalent sites).

The mutations (acidic clusters → basic clusters) carried out in SynCaMs decrease the number of sites in the main set (helix−loop−helix motifs) from four to two, whereas the number of sites in the set of moderate affinity increases from two to four. This suggests that the four helix−loop−helix motifs in the SynCaMs behave as a cooperative unit and may be modified by alteration of the electrostatic potential of the molecule.

Despite, the fact that measurents in ESI-MS are performed under conditions (pH 6, 1 mM $CaCl_2$, 20% methanol) significantly different from those used in flow dialysis (pH 7.5, 50 mM Hepes), we observe perfect agreement between these two techniques for the determination of the number of principal sites. In addition, Milos et al. (1989) have assumed that bovine brain CaM could possess up to six auxiliary sites; if we consider that SynCaM 1 is highly comparable to bovine brain CaM, five auxiliary sites are detected by ESI-MS. The observed difference of 1 may be related either to the low affinity of the class 3 sites, which hampers their exact determination by using dialysis techniques, or to a modification of the auxiliary site number due to the medium used.

It is clear that the supplementary Ca^{2+} in regard to those placed in the main sites cannot be related to nonspecific binding owing to the similar behavior of the four SynCaMs and to the good agreement between the values determined here and those previously reported by Milos et al. (1986, 1989). In addition, it must be noted that an increase in the Ca^{2+} concentration (1−5 mM) does not significantly modify the shape of the mass spectra (data not shown).

Studies of CaM$-Mg^{2+}$ complexes suggest that Mg^{2+} does not bind to the same sites that Ca^{2+} and raises the possibility of a regulatory role of these auxiliary sites. This agrees with the different requirement of Mg^{2+} and Ca^{2+} so far as the geometry of coordination is concerned. Moreover, our results show clearly that the modifications done on SynCaM 18A lead certainly to a structure very different from that of the other SynCaMs used in this study.

In this work we have shown that it is possible to deduce relevant informations about the stoichiometry of complexes between calmodulins and Ca^{2+} (or Mg^{2+}) using positive ESI-MS which appears to preserve the noncovalent interactions between Ca^{2+} and the extra sites.

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