International Journal of Mass Spectrometry 305 (2011) 87–94

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Calmodulin association with the synthetic ERα17p peptide investigated by mass spectrometry

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ARTICLE INFO

Article history:
Received 16 March 2010
Received in revised form 24 June 2010
Accepted 24 June 2010
Available online 8 July 2010

Keywords:
Calmodulin
Estrogen receptor
Non-covalent complex
Electrospray
Ion trap mass spectrometry

ABSTRACT

Implication of calmodulin (*CaM*) in breast cancer development has been proposed, justifying the interest for ER/calmodulin interaction. The association of the synthetic peptide ERα17p (H–PLMIKRSKKNSLALSLT–OH), which corresponds to the known ER interaction site of *CaM*, was investigated. Under physiologically conditions, 1:1 complex formation was observed using an ESI-ITMS instrument. This equimolar complex with *CaM* was only formed in presence of calcium. The binding of ERα17p to *CaM* is probably due to presence of four basic residues (K_{299}RSKK$_{303}$) in its sequence. Its relative binding affinity in solution by ESI-MS was evaluated by performing competitive binding experiments with other peptides more (ERα17pKR: KRSKR) or less basic (ERα17pAA: KRSAA) than ERα17p as well as Melittin (Mel), selected as a reference peptide since it is known to form a high-affinity complex with *CaM*. Relative affinities of these peptides for *CaM* were classified in the following decreasing order: Mel > ERα17pKR ~ ERα17p ≫ ERα17pAA. Interestingly, another ion series showing two peptides for one protein was detected. The specificity of this complex not often reported for Melittin is discussed.

1. Introduction

Implication of estrogens and their receptors in growth, differentiation and function of a variety of target tissues is now well established [1]. The estrogen receptor α (ER) is the dominant isoform expressed in breast cancer and contributes to the pathogenesis of breast tumors [2,3]. Calmodulin (*CaM*), an ubiquitous Ca^{2+} sensor protein, is a co-regulator which can modulate ER biological activities by inducing diverse transient complexes [4–7].

Implication of *CaM* in breast cancer development has recently been proposed, stressing interest for the complex ER–*CaM* [8]. In fact, *CaM* is an important determinant of ER-mediated transcription [9] since it has been reported to protect ER against proteasomal degradation. The 17 amino acid residues epitope P_{295}LMIKRSKKNSLALSLT_{311} of the N-terminal part of the estrogen-binding domain of ER was identified as its *CaM* binding site. Hence, by interacting with the P_{295}-T_{311} sequence, *CaM* may contribute to the orchestration of an exchange process between co-regulators implicated in ER activity. One may also assume that Ca^{2+} oscillations may modulate the cyclic profile of ER transactivation processes [10].

To further explore the mechanisms by which *CaM* cooperates with ER in the regulation of gene expression, the pharmacology of a synthetic peptide that corresponds to the *CaM*-interacting domain (ERα17p, H–PLMIKRSKKNSLALSLT–OH) was studied. Furthermore, it has been shown that exposure of breast cancer cells to this peptide provokes estrogenic responses [11].

By highlighting such an unknown ligand-independent ERα mode of activation, ERα17p provides conceptual basis for the future development of analogs capable of modulating ERα transcriptional activity and new guidelines for the design of drugs targeting ERα-mediated processes [12].

CaM forms tight complexes with a panel of proteins, in both Ca^{2+}-dependent and Ca^{2+}-independent manner [13,14]. The structure of *CaM* consists of two globular domains connected by a flexible α-helical linker [15]. Each globular domain contains two helix–loop–helix (EF hand) motifs, which are expected to be the main calcium binding sites. High-affinity binding of different targets for *CaM* is promoted by the α-helical linker [16] and several methionine residues, which are essential for folding and interaction processes [17,18]. Nevertheless, molecular mechanisms by which ERα17p associates with *CaM* remain unclear and need extensive investigations.

Among the different techniques appropriate for the investigation of non-covalent systems, electrospray ionization (ESI) mass spectrometry has numerous advantages, although it was shown

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doi:10.1016/j.ijms.2010.06.029

that non-covalent complexes could successfully be detected by using MALDI if matrices such as DHA < THA and ATT that have a pH range of 5–7 are used [19,20]. ESI (and MALDI using matrices with a pH higher than 3.5) allows non-covalent complex ionization under native conditions, by maintaining their conformation and by controlling the transfer of internal energy [21–27]. For instance competing ligand binding can be performed if they present different masses [28]. ESI-MS was used to obtain information on specificity and stoichiometry of non-covalent macromolecular assemblies [21]. Both low and high resolution electrospray-mass spectrometry has been used to study the properties associated with the binding of calmodulin with peptides, such as Melittin [29] or between other biological epitopes and synthetic peptides (RS20 [30], truncated MD myosin VI [31], nNOS [32]). In the same context, several researchers have shown that it is possible to determine relative binding affinities by ESI-MS [33–37] even if the strength of non-covalent interactions are modulated during ESI evaporation/desolvation processes. In this regard, it is noteworthy that electrostatic interactions including hydrogen bonds and salt bridges are strengthened in the absence of solvent, while hydrophobic interactions become very weaker [38,39]. Accordingly, non-covalent complexes are maintained during their transfer from solution to gas phase.

In the present investigation, ESI-MS was employed to study interaction between *CaM* and ERα17p as well as between *CaM* and two analogues with potent distinct binding properties [11]. The binding of Melittin to *CaM* was used as a reference system for evaluation of the relative binding affinity of these peptides.

2. Experimental

2.1. Materials

CaM from bovine testes and Melittin from honeybee venom were purchased from Sigma. *CaM* is N-acetylated and contains a trimethylated lysine in position 115. Prior to ESI-MS measurements, protein stock solution was desalted and buffer exchanged against 10 mM NH_4OAc (pH 6) by ultrafiltration using Microcon 10 kDa cartridge (Amicon). *CaM* was diluted in 10 mM NH_4OAc to reach a concentration of 1 mg mL^{-1} (stock solution). Even if calmodulin was already in its holo form, $CaCl_2$ was added to the solution (typically 20 mM) to compensate Ca^{2+} losses occurring during desalting step. The formation of Ca_4*CaM* was controlled by ESI mass spectrometry. Ammonium acetate and calcium chloride were obtained from Fluka. All stock solutions and buffers were prepared with 18.2 MΩ deionized water (MilliQ, Millipore).

2.2. Peptides synthesis

The peptides H–PLMIKRSKKNSLALSLT–OH (molecular mass: 1899.13 u) (ERα17p) and the two analogs H–PLMIKRSKRNSLALSLT–OH (molecular mass: 1927.14 u) (ERα17pKR) and H–PLMIKRSAANSLALSLT–OH (molecular mass: 1785.02 u) (ERα17pAA) were synthesized in solid phase by using Boc strategy (preloaded PAM resin) on an automatic peptide synthesizer 433A (Applied Biosystems) and purified by RP-HPLC (4.6 mm × 300 mm, 5 μm particle size, 300 Å pore size). All protein molecular masses of (and protein complexes) are given as average values whereas peptides masses (<5000 Da) are given as monoisotopic values.

2.3. ESI-ITMS mass spectrometry

All experiments were performed in negative or positive ion modes by using an ion trap mass spectrometer (Esquire 3000, Bruker, Bremen, Germany) equipped with an orthogonal ESI source. Samples were injected into the ESI source at a flow rate of 200 μL h^{-1}, using a syringe pump. Nitrogen was used as both nebulizing (6 psi) and drying gas (200 °C, 5 L min^{-1}). ESI mass spectra were recorded using *standard* analytical scan (m/z 50–m/z 3000) with resonant ion ejection at $\beta_z = 2/3$ ($q_z = 0.78$). The injection low mass cut-off (LMCO) was optimized to allow an efficient trapping of the high m/z range (LMCO > 95 Th). ESI mass spectra were recorded with variable values of both skimmer and capillary exit (CE) voltages to optimize non-covalent complex abundance and to avoid in source dissociation processes. Our ESI source presenting two skimmers, the first zone of desolvation was tuned by the ΔV offset between the transfer capillary exit and the first skimmer while the second zone was tuned by the ΔV offset between the first and the second skimmer. Each ΔV values were optimized to maintain intact non-covalent complexes by using potential values following: CE = −110 V, skimmer 1: −30 V and skimmer 2: −8 V. Automated ion charge control (ICC) was set to 20,000 in order to avoid space charge effect. The relative abundances of various coexisting complexes were determined from deconvoluted spectra generated by Bruker Daltonic DataAnalysis 3.3 software. For all experiments, we assumed that ion transmission efficiency was similar for the complexes and free calmodulin since charge state distributions (and therefore m/z range) of free and bound proteins were almost the same.

3. Results and discussion

3.1. ESI mass spectra of calmodulin

CaM samples were prepared using ammonium acetate buffer without organic solvent (*i.e.*, acetonitrile or methanol), in order to record mass spectra under physiological conditions. *CaM* being an acidic protein with an isoelectric point of ∼4, it would be logically negatively charged at physiological pH values. Non-specific metal ion attachments (*e.g.*, Na^+ and K^+) were reduced and a stronger protein complex signal was obtained in negative mode when compared to positive mode [40]. According to this statement, the protein has been analyzed in negative ion mode. In fact, the detection of *CaM* in the positive ion mode was less favourable displaying abundant multiple adduct ions even after extensive buffer exchange. In the present study, the negative ion ESI mass spectrum of *CaM* (Fig. 1a) showed, after purification, a multiply charged distribution state. This envelope included species containing *CaM*-derived ions presenting 2, 3 and 4 calcium ions (inset of Fig. 1a). The main ion distribution corresponds to species presenting two Ca^{2+} ions. The most abundant ion, at m/z 2107.3, was attributed to $[Ca_2CaM\text{-}13H]^{8-}$ in agreement with the calculated value (m/z 2107.33). The theoretical m/z value was determined using the following equation: $[CaM(n + 1 + 2m)H + Ca_m]/n$ where n is the global net charge of the complex ion, m is the number of Ca^{2+} ions and H is the mass of proton. The theoretical average mass of *CaM* was 16791.57 Da, as calculated from the amino acid sequence. It should be noted that one positive charge was due to the presence of a trimethyl-lysine residue in position 115. Note that the addition of one Ca^{2+} corroborates an increase of 38 u of the apo *CaM* molecular mass. A loss of two protons from *CaM* on the incorporation of each divalent metal ion was also observed [41]. Under such conditions, a distribution of species with different Ca^{2+} amount was observed, suggesting that Ca^{2+} ions were removed during *CaM* desalting. In a second experiment, an excess of Ca^{2+} ions was added to *CaM*. Fig. 1b shows the negative ESI spectrum obtained under such conditions. The charge envelope was centered around m/z 2117.0 corresponding to $[Ca_4CaM\text{-}17H]^{8-}$, where ions are observed in the range $-11 \leq z \leq -7$. Only one distribution of *CaM* with 4 Ca^{2+} ions was generated, as shown in the enlargement of the −8 charge state species (inset of Fig. 1b). This indicated that *CaM* adducted 4 Ca^{2+}.

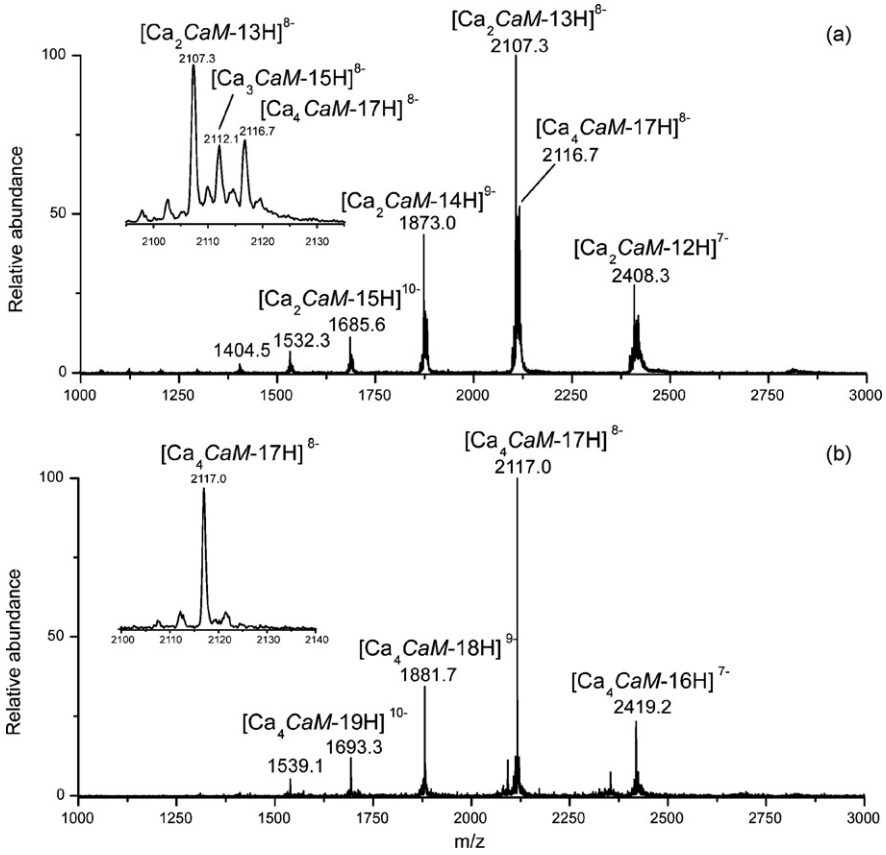

Fig. 1. ESI mass spectra of *CaM* (10 μM) in 10 mM ammonium acetate buffer (a) without and (b) with the addition of 5 equiv. of $CaCl_2$. Insets correspond to an enlargement of the −8 charge state signals.

Concerning the experimental conditions of the ESI-ion trap mass spectrometer, the declustering potential and injection low mass cut-off were optimized to allow an efficient transfer and trapping of the high *m*/*z* ions. With a LMCO value of 95 Th (for high *m*/*z* ion storage) and an excess of $CaCl_2$, ion relative abundances deduced from ESI mass spectra reflected the stoichiometry of the species present in solution ($4Ca^{2+}$ per molecule of protein).

3.2. Calmodulin–ERα17p interaction

A negative ESI-ITMS spectrum obtained after the addition of ERα17p to *CaM* in 10 mM ammonium acetate solution is shown in Fig. 2a. Molar ratios of *CaM*, ERα17p and $CaCl_2$ were 1/2/5, respectively. Under these conditions, the main ion series corresponded to the peptide addition to Ca_4*CaM* (Fig. 2b). The complex was detected over the *z* range from −7 to −12 and the charge distribution was centred on −9 charge state. Interestingly, another ion series showing a complex with two peptides per protein was detected. This Ca_4*CaM*/$(ER\alpha17p)_2$ species were produced over a very narrow charge distribution (from −8 to −10) suggesting that the folded conformation of Ca_4*CaM* is preserved. However, free cationized *CaM* (Ca_4*CaM*) was observed in significant amount. Accordingly, the free deprotonated peptide was detected at *m*/*z* 1899.4 $[ER\alpha17p\text{-}H]^-$ and *m*/*z* 949 $[ER\alpha17p\text{-}2H]^{2-}$. To allow the highest yield of Ca_4*CaM*/$(ER\alpha17p)_2$ complex, relatively hard declustering conditions (*i.e.*, the ΔV offset between the transfer capillary exit and the first skimmer potential was 80 V) and higher ion injection low mass cut-off (*i.e.*, 110 Th) were used, in order to store more efficiently the high *m*/*z* species. Thus, three *CaM* species were observed, corresponding to Ca_4*CaM* and Ca_4*CaM* bound to one or two peptides. Deconvolution of all charges gave a *CaM* plus 4 Ca^{2+} mass at 16,943 Da and a Ca_4*CaM* complex with 1 or 2 peptides at 18,843 Da and 20,742 Da, respectively. All species were observed with a relatively lower average charge state and a narrow distribution consistent with a folded native conformation in solution, where less acidic residues are available for deprotonation, as shown in Fig. 2a.

It should be stressed that the attachment of two peptide ligands to *CaM* is not common, although already observed [42–44]. This second peptide interaction could be related to a non-specific association.

3.3. Optimization of calmodulin–ERα17p complex detection

3.3.1. Desolvation zone

To detect non-covalent interactions, appropriate desolvation conditions in the ESI source should prevent complex dissociation process. Indeed, a threshold exists between the energy required to produce gas phase desolvated protein complex ions and the energy resulting in complex ion dissociation. One of the critical energy parameters for producing intact gas phase ions is the voltage applied to the transfer capillary and skimmers (in ESI-ion trap interface). In our ion trap mass spectrometer, 2 skimmers are present in the desolvation zone. Desolvation conditions were controlled by changing the ΔV values between the transfer capillary exit and the first skimmer and also by modifying the ΔV value between the first and the second skimmer. Fig. 3 shows relative abundance changes as a function of the skimmer 1 potential. In the latter case, potential difference in the first zone was maintained constant by raising the potential of the transfer capillary exit in the same way, whereas the potential difference in the second zone increased.

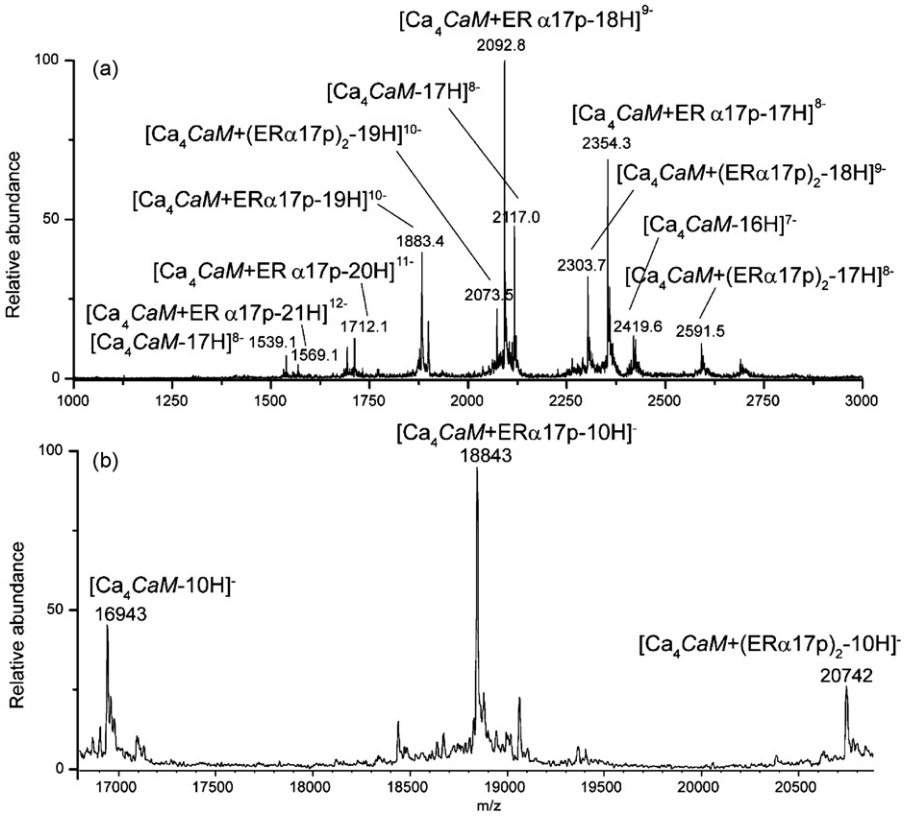

Fig. 2. (a) ESI-MS spectrum of a 10 μM solution of *CaM* with 20 μM of $CaCl_2$, 50 μM of ERα17p, in 10 mM of ammonium acetate and recorded in the negative ion mode. (b) The deconvoluted spectrum.

Under these desolvation conditions, Ca_4*CaM*/ERα17p complex was produced as the base peak at low skimmer 1 potential (in absolute value). In contrast, at higher skimmer 1 potential values corresponding to harder desolvation conditions, the relative abundance of [Ca_4*CaM* + P] and [Ca_4*CaM* + 2P] species decreased. When the skimmer 1 potential was set to −50 V, free calmodulin species were produced as base peak. At −80 V no protein/peptide complexes were detected, suggesting a complete dissociation of the later. In terms of relative abundance, the most appropriate conditions to reach calmodulin/peptide complex were obtained with skimmer 1 value lower than −20 V. Nevertheless, in such very soft desolvation conditions, the sensitivity was particularly low. Thus, in order to produce and transfer intact protein complex ions with good yield (*i.e.*, absolute abundance), energy compromise was defined in such a way that it produced a maximum number of intact complex ions. Studies were realized with a skimmer 1 set to −30 V. Whatever energy values, the number of Ca^{2+} was constant, suggesting that the association between *CaM* and Ca^{2+} corresponded to very stable species.

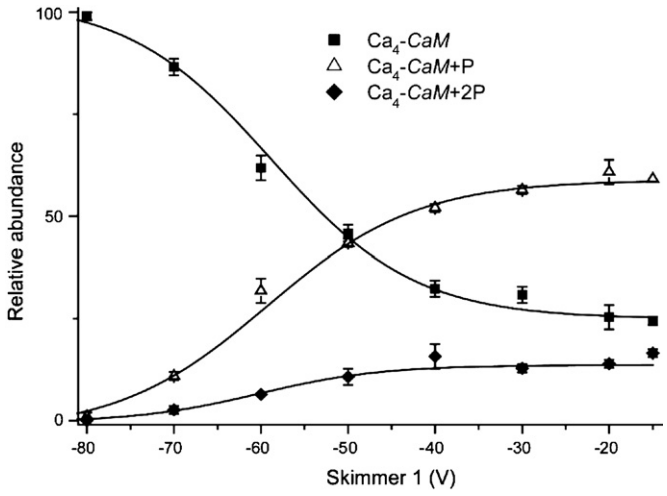

Fig. 3. Influence of skimmer 1 potential on signal intensity of Ca_4*CaM*–peptide and Ca_4*CaM*–2 peptide complexes.

3.4. Specificity of the calmodulin–ERα17p interaction

CaM binds to a number of small amphiphatic peptides in a calcium-dependent manner [45]. Previous data showed that four Ca^{2+} ions were preliminarily bound to *CaM* before peptide association (Figs. 1 and 2). To determine the role of Ca^{2+} ions in Ca_4*CaM*/peptide complex, a series of experiments consisting in stepwise Ca^{2+} removal was carried out (Fig. 4). In the presence of a weak excess of Ca^{2+}, the complex was promptly saturated, yielding principally Ca_4*CaM*/ERα17p. In this regard, no Ca^{2+} stoichiometry higher than 4 was observed, suggesting that the complexation of Ca^{2+} to *CaM* was specific. In additional experiments, apo-calmodulin was produced in the presence of EDTA. Thus, we evaluated the strength of the *CaM*–Ca^{2+} interaction and we explored whether the interaction between *CaM* and ERα17p was also observed with apo-calmodulin.

Remarkably, with a relatively high amount of EDTA (75 μM), apo-calmodulin was exclusively observed (Fig. 4a). In this condition, the amount of *CaM*/ERα17p complex was marginal, confirming definitively that Ca^{2+} is required for the formation of the complex. With lower amount of EDTA (50 μM) two Ca^{2+} ions were easily removed from the complex whereas the two other remained tightly bound (Fig. 4b). It is noteworthy that the most abundant complex shares a charge of −8, suggesting that the secondary

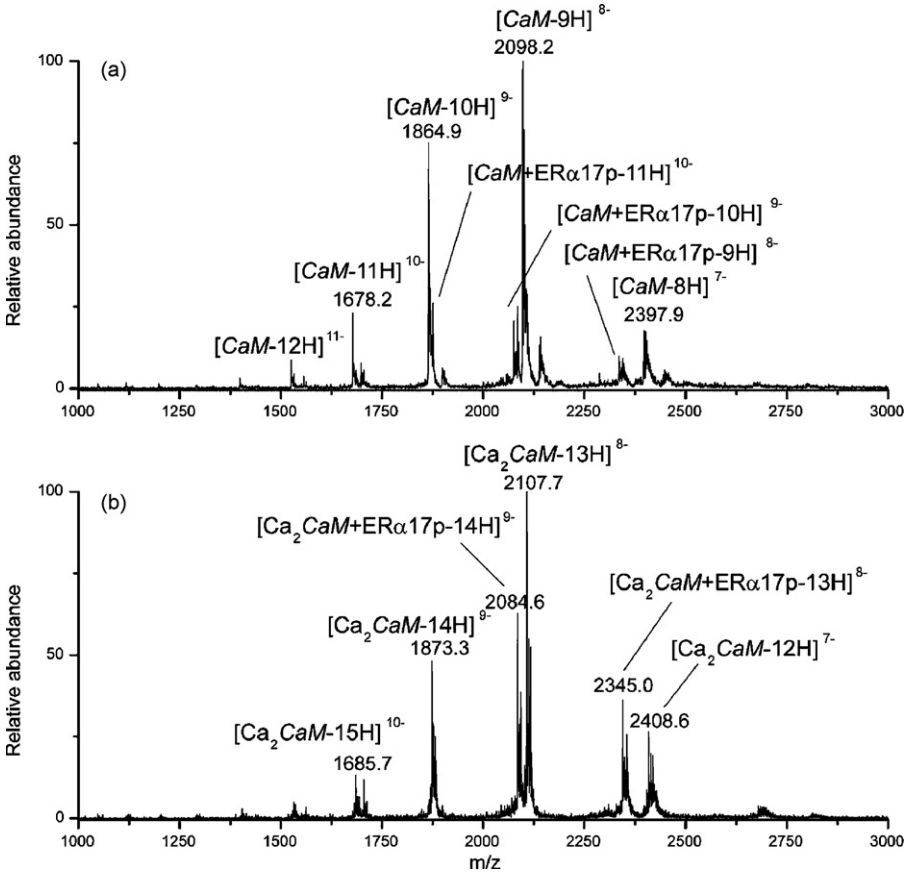

Fig. 4. Influence of Ca^{2+} removal on formation of *CaM*–peptide complexes using (a) 75 μM EDTA and (b) 50 μM EDTA.

structures of separated *CaM* domains in complex with ERα17p are similar to those observed with Ca_4*CaM* [46,47]. Note that complex with one or three calcium ions were not observed, confirming the specificity of interaction. These data are consistent with previous studies that evidenced cooperative effects in Ca^{2+}-binding [48] Interestingly it is observed that the association of ERα17p to *CaM* is very calcium dependent. Peptide/protein complex was produced in significant amount in presence of 2 Ca^{2+} ions (Fig. 5). In presence of 4 Ca^{2+}, the one peptide complex was the most abundant species while the two peptides complex became significant.

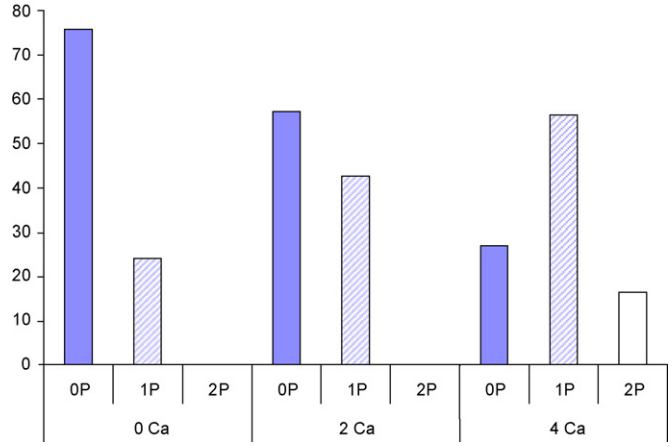

Fig. 5. Evolution of the association of ERα17p to *CaM* as a function of the number of complexed Ca^{2+} (relative ion abundance in % determined from the summation of all charge state of each species).

Finally, the fact that well-defined stoichiometries were observed, demonstrated that 2 and 4 Ca^{2+} binding are specific. This is in total agreement with the known behaviour of *CaM* toward Ca^{2+} and its ability to bind two Ca^{2+} at each of its two lobes [49,50].

3.5. Stability of the complexes: competition reactions

The binding of ERα17p peptide to *CaM* in the presence of calcium is strongly related to the presence of four basic residues in its sequence. Indeed, it is well known that strong electrostatic interactions occur between the acidic motifs of *CaM* and basic residues of peptides [44]. The stabilizing properties of the basic $K_{299}RSKK_{303}$ motif were investigated when ERα17p is in mixture with other peptides (in equimolar concentration) and in presence of *CaM* (10 μM) and $CaCl_2$ (15 μM).

First, the strength of interaction between Ca_4*CaM* and modified ERα17 peptides, *i.e.*, ERα17pKR (more basic than ERα17p) or ERα17pAA (less basic than ERα17p), was studied. Owing to its higher amount of basic residues, ERα17pKR is expected to present a higher affinity to Ca_4*CaM*. The relative abundance of the [Ca_4*CaM*–ERα17pKR] complex displayed in the mass spectra was only slightly higher (4%, evaluated from the charge deconvolution spectrum) than [Ca_4*CaM*–ERα17p] (Fig. 6a). In this context, it is outlining that the ER mutant $K_{303}R$ is observed in a number of breast tumors [51,52]. When the relative abundances of the [Ca_4*CaM*–ERα17pKR] and [Ca_4*CaM*–ERα17p] species were compared in function of charge state, we observed that the abundance of the former increased slightly when the charge state increased. This could be mostly due to the differences of chemical properties (acidity, basicity, hydrophobicity, etc.) of arginine, yielding different ionization efficiency. In the case of ERα17pAA (Fig. 6b),

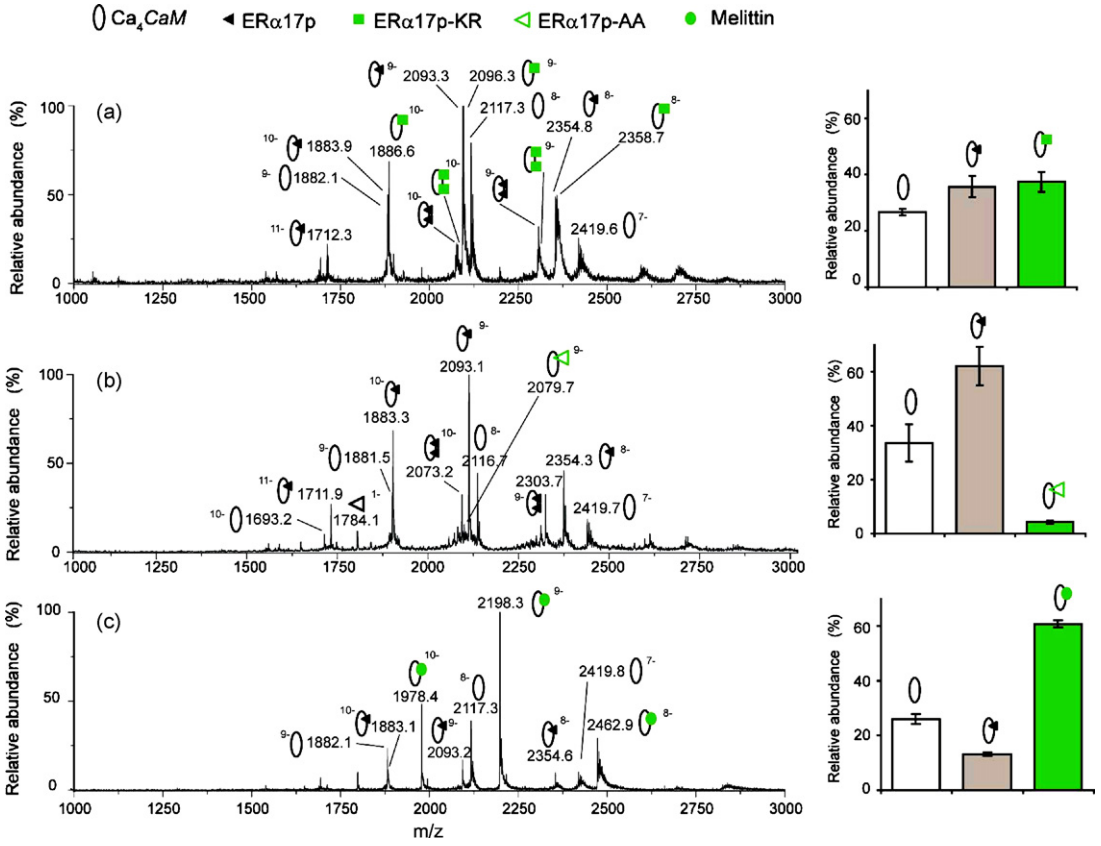

Fig. 6. ESI mass spectra calmodulin (10 μM) with ERα17p in 1:1 mixture with (a) ERα17pKR (50 μM), (b) ERα17pAA (50 μM) and (c) Melittin (10 μM). Histogram for each mass spectrum represents the relative abundance of complexes after deconvolution. Error bars correspond to standard deviation obtained for 3 replicates.

the relative abundance of the [Ca_4*CaM*–ERα17pAA] complex was strongly reduced, confirming the importance of basic residues for the association between ERα17p and *CaM* [8]. In this regard, it should be stressed that the [Ca_4*CaM*–ERα17p-18H]$^{9-}$ ion (*m*/*z* 2093.1) corresponded to the base peak whereas the ion corresponding to [Ca_4*CaM*–ERα17pAA-18H]$^{9-}$ (*m*/*z* 2079.7) was detected in weaker abundance (~10%). In addition, [Ca_4*CaM*-17H]$^{8-}$ (*m*/*z* 2116.7) was detected with an abundance of 40%, suggesting that [Ca_4*CaM*–ERα17p] was preferred. Hence, the two basic residues K302 and K303 of ERα17p are of prime importance in its association with Ca_4*CaM*, an observation that is relevant to its stabilization through electrostatic interactions rather than hydrophobic interaction. More precisely, the two lysine residues being very basic it is possible that the complex may be preserved through salt bridge formation for the detection in gas phase.

Our data were compared to those recorded with Melittin (Mel, 2846.5 Da) used as a reference [53,54]. The [Ca_4*CaM*–ERα17p-18H]$^{9-}$ ion (*m*/*z* 2093.6) was about a sixth of the peak corresponding to [Ca_4*CaM*–Mel-18H]$^{9-}$ ion (*m*/*z* 2198.3), suggesting that the affinity of ERα17p for Ca_4*CaM* was significantly lower than that of Melittin (Fig. 6c). Accordingly, relative affinities of tested peptides were as followed (decreasing order): Mel > ERα17pKR ~ ERα17p > ERα17pAA. Interestingly, affinities were proportional to the number of basic residues.

3.6. Influence of peptide concentration

The influence of ERα17p concentration on the relative abundance of the complex Ca_4*CaM*–ERα17p was evaluated (Fig. 7a). This experiment was performed with ERα17p (Fig. 7a) and with Melittin as reference (Fig. 7b). In both cases, the concentration of *CaM* was 4 μM. As expected, ERα17p concentration had important effects on the relative abundance of the complexes pre-formed in solution. Free calmodulin was produced as the base peak up to 4 μM of ERα17p. From 4 μM, the Ca_4*CaM*–ERα17p complex corresponded to the base peak in the mass spectra while from 20 μM the Ca_4*CaM*–$(ERα17p)_2$ complex was detected in significant amount (Fig. 6a). At high ERα17p concentrations, the presence of free *CaM* was relevant to the relatively low affinity of the peptide for *CaM*. However, it cannot be ruled out that complex dissociation occurred during the desolvation process, since this process was significantly different in the presence of Melittin (Fig. 7b) (higher affinity).

The effects mediated by concentration changes of Melittin on the relative abundance of the complex Ca_4*CaM*–Mel was different from ERα17p (Fig. 7b). From 4 μM, the Ca_4*CaM*–Mel complex corresponded to the base peak while beyond 10 μM, the Ca_4*CaM*–Mel complex was the only species detected. From 15 μM of Melittin, Ca_4*CaM*–Mel complex was formed in competition to Ca_4*CaM*–Mel_2, which was detected in significant amount (Fig. 7b). From about 20 μM of Melittin, the 1:2 complex was detected as the base peak. It should be noted that even with larger amount of Melittin, no association with more than two peptides was detected. The origin of the calmodulin complex with two peptides is however unclear.

In general, calmodulin binds Melittin in a calcium-dependent manner, yielding a 1:1 complex. This remark is in good agreement with our experimental results as up to about 15 μM of Melittin only the 1:1 complex is detected. The existence of this two peptides complex has been already observed by Follenius-Wund et al. using spectroscopic methods [42]. The authors found that the affinity of Melittin for Ca_4*CaM*/Melittin complex is about three orders of magnitude lower than for Ca_4*CaM*. The two peptides complex could be related to the ability of each *CaM* domain to bind Melittin [55]. Even if the association of *CaM* with two peptides is not

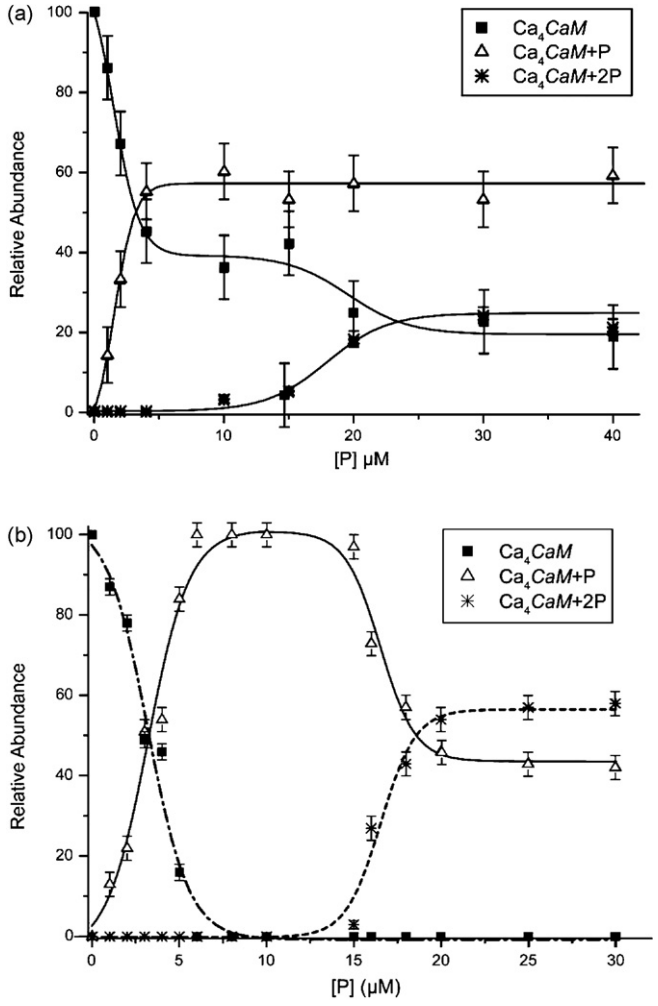

Fig. 7. Influence of peptide concentration on the signal intensity of Ca_4CaM/peptide and Ca_4CaM/$(peptide)_2$ complexes (a) ERα17p and (b) Melittin. The concentration of *CaM* was maintained at 4 μM with 20 μM of $CaCl_2$. Data points have been fitted with polynomial functions.

frequently observed, such behavior was already proposed clearly for Melittin and also detected (by using cross-linking experiments) with another peptide but it has been considered as a possible artifact [43]. Our results show that *CaM* can bind up to two peptides such as Melittin or ERα17p. If this second peptide interaction was relevant to a non-specific association, a more statistical distribution would be expected, as shown in other investigation with MALDI [44]. In the present work, no association of *CaM* with more than two peptides was detected, even with large excess of peptide (*i.e.*, 10 equiv.), suggesting that this second association is really specific.

Accordingly, in the case of Melittin, the 1:1 complex occurs in the middle of the two lobes of *CaM* and is accompanied by conformational changes. In contrast, the addition of the second peptide is accompanied by deep conformational changes. This could be consistent with the fact that the addition of the second peptide requires an important rise of the concentration. In the case of ERα17p the relatively small affinity difference between the 1:1 and the 1:2 complex suggests that the addition of the second peptide does not require such important conformation change. In fact in the case of the 1:2 ERα17p complex each peptide would be attached to each lob. This is supported by the fact that with Ca_2CaM, only association with one peptide was detected.

4. Conclusion

Our study highlights that ERα17p interacts with a stoichiometry 1:1 with Ca_4CaM. Also, ERα17p also interacts with lower affinity with Ca_4CaM yielding a rarely described 1:2 stoichiometry. This characteristic is consistent with an interaction of ERα17p with two distinct sites of distinct affinities, a process that has been confirmed with Melittin. Potential biological significance of this property is unknown although it may suggest that, under peculiar physiological/pathological conditions, *CaM* would participate to the dimerization of ER in a hormone-dependent and calcium-dependent manner, a property of prime importance for ER-mediated transcription. Nevertheless, NMR studies are necessary to explore the mode of binding of ERα17p to *CaM*. Amazingly, our work may perhaps provide a key element for the switch between cytoplasmic and nuclear signalling pathways to which ER participates and may thereby constitute the “tip of the iceberg” of estradiol-independent ER-dependent transcription activation.

Acknowledgments

UPMC and CNRS are acknowledged for financial support. Dr Amina S. Woods is gratefully acknowledged for helpful discussion.

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