Low Temperature Aqueous Electrospray Ionization Mass Spectrometry of Noncovalent Complexes

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In the present study we describe conditions that permit the characterization of noncovalent protein–substrate complexes in aqueous solution by microspray electrospray ionization-mass spectrometry (ESI-MS), using a heated transfer capillary at low temperature (45 °C). Specifically, we examined the binding of calmodulin to two polypeptides; the calmodulin-binding domain of calmodulin-dependent protein kinase II (CamK-II) and melittin. Calmodulin, a well known calcium-binding protein, binds to a number of small amphipathic peptides in a calcium-dependent manner. Our results directly show that both peptides form equimolar complexes with calmodulin only in the presence of calcium. The stoichiometry necessary for the formation of each complex was 1:1:4 for calmodulin:peptide (melittin or CamK-II):Ca^{2+}, respectively. Furthermore, it is demonstrated that the detection of the complex in ESI-MS is source temperature dependent. (J Am Soc Mass Spectrom 1998, 9, 580–584) © 1998 American Society for Mass Spectrometry

Recently, electrospray ionization-mass spectrometry (ESI-MS) has been used to investigate numerous noncovalently bound supramacromolecular complexes. Some of the different complexes observed with ESI-MS involve protein–protein [1, 2], protein–ligand [3–5], protein–oligonucleotide [6, 7], and protein–double stranded DNA noncovalent interactions [8]. The application of ESI-MS in this area has also recently been the subject of an extensive review [9]. The ESI process produces gas phase macromolecular ions directly from solution. Typical ESI-MS operational conditions, however, are often not compatible with the detection of noncovalent protein–substrate complexes [9]. Optimal ESI stability and sensitivity is usually achieved by the addition of an organic modifier to the sample solution, thereby reducing its surface tension and aiding aerosol formation [10]. In addition, some commercially available interfaces use a capillary that is typically heated to >150 °C to promote desolvation and ion transfer into the gas phase. Thermal decomposition of the complex, however, is also likely under these conditions. Therefore, as previously mentioned by Loo et al. [1], detection of a noncovalent supramolecular complex often relies on a compromise between the "softness" of the ESI ionization conditions and the required instrument sensitivity. In addition, direct analysis of complexes dissolved in physiologically relevant solutions, in particular aqueous solutions, is highly desirable. Indeed, Loo [9] recently noted that only a very limited number of noncovalent complexes derived from aqueous solution have been detected. However, the emergence of microspray and nanospray interfaces have improved the potential for analyzing such solutions. Furthermore, the use of a "cooled" interface for these types of studies is becoming a reality [3].

Previously Gross and co-workers [10] demonstrated using conventional ESI conditions that calcium (Ca^{2+})-loaded calmodulin binds the polypeptide melittin. In this study we show that similar results are obtained using a low-temperature microspray ESI setup for the direct analysis of aqueous solutions of the Ca^{2+}-loaded protein/peptide supramolecular complex. During these studies we also investigated the interaction of calmodulin with the calmodulin binding peptide of calmodulin-dependent protein kinase II (CamK-II). Here we report our approach and show the detection of supramolecular complexes by ESI-MS directly from aqueous solution. In addition, we confirm the Ca^{2+} dependency of the calmodulin/peptide interaction [11] and readily determine the binding stoichiometries of all elements of the noncovalent complex.

Experimental

Materials

Bovine brain calmodulin, melittin, CamK-II, calcium acetate, and magnesium acetate were purchased from

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Sigma (St. Louis, MO) and used without further purification. Protein and peptide samples were dissolved in H_2O at a concentration of 20 μM and pH of 6.1. Protein/peptide complexes were prepared by mixing the protein and peptide solutions to the final concentrations given in the figure legends. Samples were allowed to equilibrate for 20–30 min before data acquisition. For the experiments measuring the Ca^{2+}-dependent binding of melittin and CamK-II to calmodulin, calcium acetate was added to apo-calmodulin already dissolved in H_2O. Addition of calcium acetate (total ~100 μM) resulted in a final pH of 5.7.

ESI-MS

ESI-MS measurements were recorded on a Finnigan MAT 900 mass spectrometer (Bremen, Germany), using a modified microspray ESI introduction interface [12]. ESI measurements were acquired in both positive and negative ion mode. Protein solutions were introduced into the ESI source via 50 μM i.d. fused silica, from a 50 μL syringe, using a Harvard Apparatus Model 22 syringe pump (South Natick, MA), at a flow rate of 0.5 μL/min. Sulfur hexafluoride was introduced through the auxiliary gas port at approximately 2 μL/min, to prevent formation of corona discharge in negative ion mode. The ionization source and heated capillary were set to a temperature of 30 °C (the ambient temperature of both during operation measured ~45 °C). An instrument resolution of ~1000 was used for all analyses. The scan range was 500 to 3500 Da at a rate of 30 s per decade. The position and time resolved ion counter (PATRIC) focal plane array detector was used throughout at a voltage of 750 V and a mass window of 8%. Multiple scans were recorded and summed by the instrument data system (Finnigan MAT ICIS software, version 8.01HB). Multiply charged spectra were transformed to obtain the M_r value using algorithms supplied with the instrument data system. For ESI-MS measurements, the protein and protein/peptide mixtures were sprayed into the ESI source region in H_2O. Concentrations of calmodulin (20 μM), CamK-II, and melittin (20–100 μM), calcium acetate (100 μM), and magnesium acetate (100 μM) were used.

Results

Initial reports by Gross [10] indicated that ESI-MS analysis of a Ca^{2+}-loaded calmodulin–melittin mixture, employing organic solvent in the spray revealed a 1:1 protein–peptide complex. Concurrently, we were also investigating formation of this complex using a low temperature, aqueous, micro-ESI system to study such protein–peptide noncovalent interactions. The addition of melittin (20–100 μM) to a solution of Ca^{2+}-free calmodulin (20 μM), at pH 6.1 did not produce any detectable evidence of protein/peptide complex formation (Figure 1A). However, addition of Ca^{2+} (in the form of calcium acetate, 100 μM) to the protein/peptide solution resulted in the calcium dependent formation of a calmodulin/melittin complex (Figure 1B). Transformation of the data revealed that predominantly four Ca^{2+} ions were bound to calmodulin (data not shown). It should be noted that addition of 100 μM calcium acetate to the unbuffered protein/peptide solution resulted in a small change in pH to 5.7. However, in a previous fluorescence study [13] examining the efforts of pH on the conformation of calmodulin showed that in the pH range 5–6.5, no pH-induced conformational change of the protein occurred. We also detected a significant shift to a lower charge state distribution in the negative ion ESI-MS multiply charged spectrum upon formation of the Ca^{2+}-loaded calmodulin–melittin complex (see later). This shift in charge state distribution was also noted by Gross [10]. However, we only detected complex formation when using a cooled ESI interface at a temperature of ~45 °C. Furthermore, addition of Mg^{2+} to calmodulin resulted in metal ion uptake but no detectable change in charge state distribution nor complex formation (data not shown). These

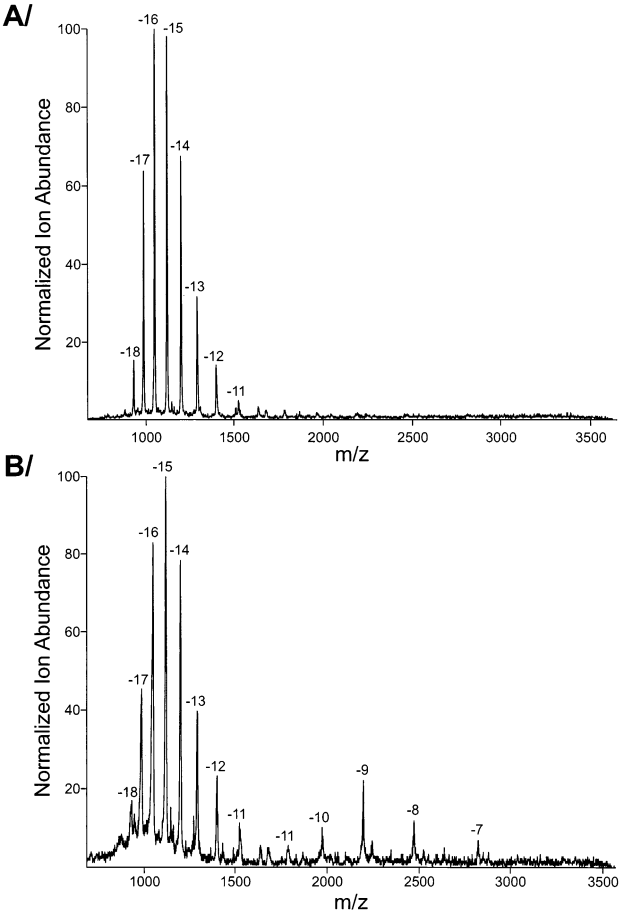

Figure 1. (**A**) Negative ion ESI-MS of calmodulin (50 μM) in the presence of melittin (50 μM), pH 6.1. (**B**) Negative ion ESI-MS of calmodulin (50 μM) in the presence of melittin (50 μM) and 100-μM calcium acetate, pH 5.7. Labeled ions representing uncomplexed calmodulin are observed between m/z 900 and 1600 and labeled ions representing the complex are detected between m/z 1700 and 3000.

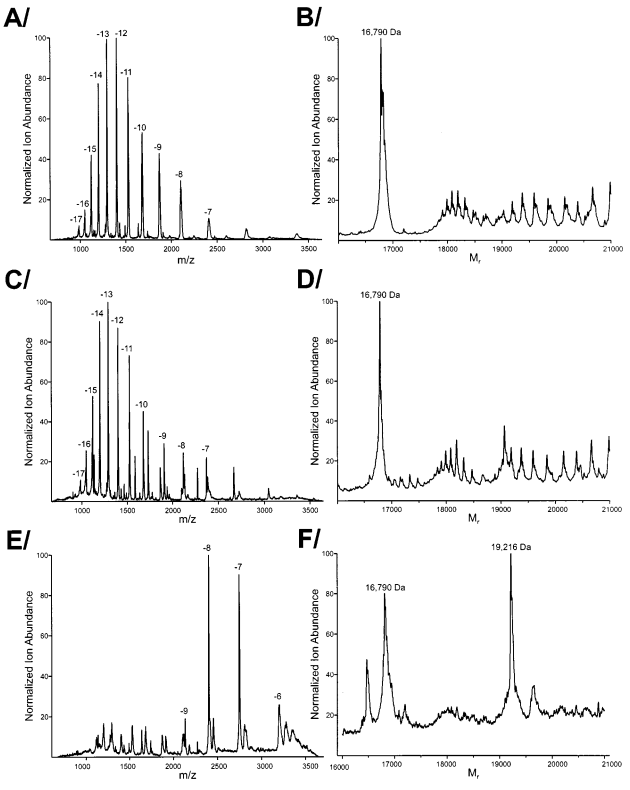

Figure 2. (**A**) Negative ion ESI-MS and (**B**) transformed spectrum of apo-calmodulin dissolved in H_2O, pH 6.1. (**C**) Negative ion ESI-MS and (**D**) transformed spectrum of calmodulin (50 μM) in the presence of CamK-II (50 μM), pH 6.1. (**E**) Negative ion ESI-MS and (**F**) transformed spectrum of calmodulin (50 μM) in the presence of CamK-II (50 μM) and 100-μM calcium acetate, pH 5.7.

results are in agreement with other spectroscopic studies which show that Mg^{2+} binding does not induce a significant conformational change within calmodulin [14, 15].

In a second series of experiments we examined the Ca^{2+}-dependent formation of the complex between calmodulin and CamK-II. Again, our ESI interface was used at ambient temperature during initial studies of the complex. The multiply charged and transformed spectra of an aqueous solution of apo-calmodulin (pH 6.1) obtained under these conditions are shown in Figure 2A, B. The transformed spectrum (Figure 2B) shows a major ion response at M_r = 16,790 Da which matches the expected molecular mass of Ca^{2+}-free calmodulin. Another response corresponding to addition of 1 Ca^{2+} to calmodulin was also detected and results in a slight shift in charge-stage distribution compared to apo-calmodulin shown in Figure 1A. The ion responses observed between M_r = ~18,000–21,000 Da appear to be either contaminants present in calmodulin or nonspecific adducts. Addition of CamK-II to this calmodulin solution resulted in no change in the multiply charged protein spectrum (Figure 2C). The transformed spectrum (Figure 2D) also only shows predominantly free calmodulin at M_r = 16,790 Da, as well as the contaminants and nonspecific adducts observed in Figure 2B. Addition of CamK-II and Ca^{2+} to apo-calmodulin (pH 5.7) resulted in the appearance of four new responses in the multiply charged spectrum (Figure 2E), representing the −6, −7, −8, and −9 charge states of the Ca^{2+}-loaded calmodulin/CamK-II complex. Transformation of these responses (Figure 2F) provided a M_r = 19,216 Da which is equal to the mass of the CamK-II peptide (M_r = 2274 Da) plus calmodulin bound 4 Ca^{2+} (M_r 16,942). These results confirm the Ca^{2+} dependency for the binding of CamK-II to calmodulin as noted by other workers [11]. Furthermore, the ability to detect complex specific responses only in the presence of Ca^{2+} proves that these responses do not result from ion/molecule reactions that may occur within the ESI interface but are specific and characteristic of the composition of the bulk solution.

As previously mentioned, the initial calmodulin/CamK-II experiments were conducted with the temperature of both heated ESI transfer capillary and mass spectrometer focusing lenses set to 30 °C (actual measured temperature of both was 45 °C). A fine line exists between the energy input required in order to achieve gas phase protein complex ions and that energy which produces ions but results in the dissociation of the complex. Two of the critical energy parameters (in our interface) for producing gas phase ions are the temperature of the heated capillary and the voltage applied to the heated capillary, tube lens, and skimmer. To investigate the effect of the ESI source temperature on the ability to observe protein/peptide complexes, we recorded the negative ion ESI-MS spectrum of the calmodulin/CamK-II complex at a fixed focus lens temperature (50 °C), but with various temperatures applied to the heated ESI-MS transfer capillary (Figure 3A, B). The complex was observable at 30 °C (Figure 3A) and 80 °C (Figure 3B), however increasing the temperature to 120 °C severely decreased the amount of complex detected (data not shown). At 180 °C, although superior ESI-MS sensitivity was achieved, the complex responses were much smaller, and the spectrum was dominated by peaks representing singly and doubly charged CamK-II peptide ions, as well as CamK-II oligomers (Figure 3C).

Discussion

The results presented above show the potential power of ESI-MS for the analysis of protein/peptide complexes. ESI-MS offers many advantages over other techniques used to characterize macromolecular complexes. In particular, as shown in the current study, ESI-MS analysis provides direct evidence of the Ca^{2+}-dependent formation of calmodulin/peptide complex formation. Furthermore, the unequivocal stoichiometry of Ca^{2+}/calmodulin/peptide was determined to be 4:1:1 for both melittin and CamK-II bound to Ca^{2+}-loaded calmodulin. In the presence of Mg^{2+}, calmodulin took up between 4 and 6 metal ions but no protein/

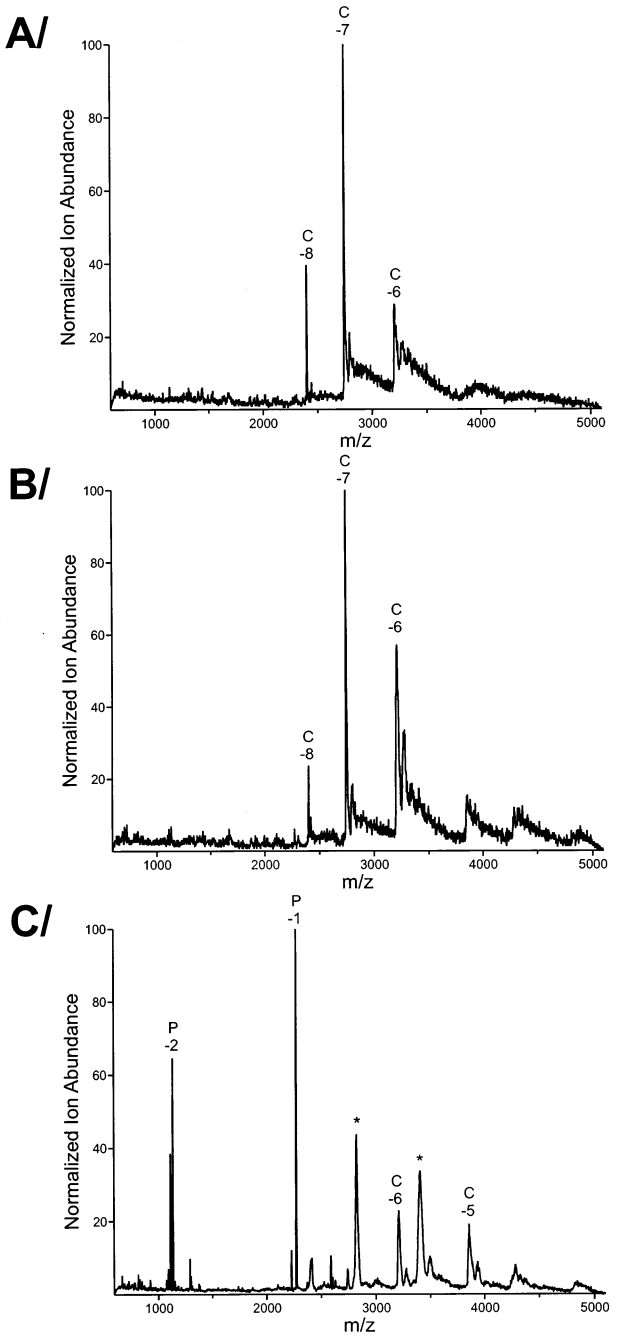

Figure 3. Negative ion ESI-MS spectra of calmodulin-Ca^{2+}/CamK-II complex acquired at a heated capillary temperature of (**A**) 45 °C, (**B**) 80 °C, and (**C**) 180 °C. The charge states representing the complex are labeled with a C, whereas those representing free CamK-II are labeled with a P. Ions corresponding to CamK-II oligomers are labeled with an asterisk.

peptide complex was observed, demonstrating the strict requirement of Ca^{2+} ion dependent complex formation. These results were obtained by analysis of the calmodulin/peptide system dissolved in aqueous solution. In previous work by Gross [10], they examined the calmodulin/melittin complex by introducing their samples through an infusion loop containing a solution of 20:80 methanol:H_2O prior to ESI analysis. It has been shown previously that the introduction of even small amounts of methanol or other organic solvents can have significant effects on protein structure and stability [16–18]. Although the amount of methanol added in analysis of the calmodulin–melittin complex did not appear to affect the structure or ability of calmodulin to bind melittin [10], the ability to acquire similar data in aqueous solution would make ESI more universally attractive to the biochemical community. In addition, in this work we were able to acquire all data with a "cooled" microelectrospray interface, in conjunction with direct aqueous spray conditions.

Previously, Gross [10] had noted that saturation of calmodulin with Ca^{2+} results in the appearance of a biomodal charge-state distribution in the negative ESI spectrum and is indicative of two different protein conformer populations. We have also observed the same phenomenon when calbindin $D_{28\,K}$ and two mutant forms of calbindin bound either Ca^{2+} or Tb^{3+} ions [5]. We also compared, using the same three proteins, the changes in their ESI-MS spectra with changes in their fluorescence and circular dichroism spectra upon binding of Ca^{2+}. These studies demonstrated that changes in charge state distribution on metal ion uptake reflect gross changes in tertiary structure of the protein [19]. However, it is interesting to note that both Gross [10] and this work show that the peptide-bound complex of Ca^{2+}-loaded calmodulin has a very similar charge state distribution to just Ca^{2+}-loaded calmodulin.

Intriguingly, this latter set of observations is consistent with the recent observation that "the backbone conformations of individual domains [of calmodulin] complexed with peptide are essentially identical to those of Ca^{2+}-calmodulin" [20]. However, it is important to note that such findings should be interpreted with care since it is also well documented that some complex localized tertiary structural change upon binding of the peptide to Ca^{2+}-loaded calmodulin does occur [21]. Furthermore, in positive ion ESI-MS, no change in charge state distribution occurs to any great extent (data not shown), and further studies are ongoing. Obviously as more protein complexes are studied the ability of ESI-MS in determining the types of interactions involved in these complexes will become clear.

Finally, in order to produce intact gas phase protein complex ions, an energy compromise must be made which produces the maximum number of ions for detection yet maintains an intact complex. In the calmodulin-Ca^{2+}/CamK-II system, increasing the heated capillary temperature above 80 °C, whereas maintaining a constant tube lens voltage, resulted in a significant degree of complex dissociation. As the use of ESI-MS expands in the study of supramolecular complexes, a greater understanding of the energy requirements needed to produce and maintain noncovalent protein complex ions will be vital. Such an understanding will

also be crucial in the potential use of ESI to measure association and dissociation constants.

In conclusion, the results presented here show the usefulness of ESI-MS in the measurement of protein–peptide complexes in aqueous solution at near physiological temperatures. ESI-MS analysis of supramolecular complexes is rapid and requires small amounts of solution and, in the cases presented above, directly provides not only the protein–peptide stoichiometry, but also the metal-binding stoichiometry required for complex formation.

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