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Characterization of Low Affinity Complexes Between Calmodulin and Pyrazine Derivatives by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry (ESIMS) was used to study the weak non-covalent interactions occurring between 6-bromo-3-(hydroxymethyl)-8-(methylamino)imidazo[1,2-*a*]pyrazine (1) and calmodulin. The formation of a 2:1 (ligand:protein) complex was observed. Using 2, a (diazomethyl)carbonyl derivative of 1 which under UV irradiation generates a highly reactive carbene entity, calmodulin was photo-labeled and the mass spectrum of the covalent adduct was recorded. Under these circumstances, two species were detected, one corresponding to the binding of calmodulin to four carbenes derived from 2 and another corresponding to calmodulin five carbenes after their loss of a bromine atom. These results strongly confirm that ESIMS is a powerful technique for the characterization of low-affinity complexes, even if part of the non-covalent interactions could be lost during the ESI process.

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INTRODUCTION

In recent years, great improvements in the biological mass spectrometry have been provided by the emergence of matrix-assisted laser desorption ionization (MALDI)[1,2] and electrospray ionization (ESI).[3] The latter is a mild ionization method allowing the molecular masses of large biological macromolecules (e.g. proteins, nucleic acids and polysaccharides) to be measured.[4–6] In addition, it is well established that ESI preserves non-covalent interactions, thus providing important information on receptor–ligand, antibody–antigen, enzyme–substrate, protein–cofactor and guest–host complexes.[7] Thus, Light-Wahl *et al.*[8] recently observed the non-covalent tetrameric active forms of avidin, concavalin A and adult human hemoglobin. However, this feature raises the interesting question of what range of affinities for the formation of non-covalent complexes are likely to be observable. We have found that the system calmodulin (CaM)–{6-bromo-3-(hydroxymethyl)-8-(methylamino)imidazo[1,2-*a*]pyrazine} (CaM–**1**) could provide some important insights into this problem.

Compound **1** is a very interesting substance exhibiting a potent smooth muscle relaxant activity.[9–12] CaM is a ubiquitous protein involved in many cellular processes, such as muscle contraction.[13,14] As shown by a microcalorimetric method, **1** binds to CaM with low affinity ($K_d \approx 0.8 \times 10^{-3}$ l mol^{-1}).[15] Therefore, we have attempted to look for the existence of associations between CaM and **1** using ESI mass spectrometry (MS). Using this technique, Loo *et al.*[16] have recently shown that it is possible to detect weak molecular interactions (millimolar range) between human angiotensin and eight synthetic antisense octapeptides. Here, we have attempted to confirm this result using not small peptides but a protein.

EXPERIMENTAL

Materials

The CaM used in this study (M_r 16627 Da) was a hybrid between plant and mammalian CaM. It was produced and purified as described previously.[17–20] Compounds **1** and **2** were synthesized as reported previously.[9–12] All solvents were purchased from SDS (Peypin, France) and reagents from Merck (Darmstadt, Germany).

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Sample preparation

A 1 mg (6×10^{-8} mol) amount of metal-free CaM[20] was dissolved in 900 μl of 5.5 mM ammonium acetate containing 1 mM $CaCl_2$ (pH 6.0, adjusted with acetic acid). To this solution was added 5×10^{-7} mol of **1** or **2** in 100 μl of methanol–water (60:40, v/v). The mixture was centrifuged for 10 min at 5000 rpm to prevent clogging of the capillary by particulates and 10 μl (100 pmol μl^{-1}) of the supernatant were injected to acquire ESI mass spectra.

Photoaffinity labeling

After 30 min of equilibration in the dark, a solution containing CaM and **2** (see above) was irradiated with a Phillips TUV6 lamp (254 nm). A 1 ml volume of the mixture was stirred at a distance of 5 cm from the ultraviolet light source for 30 min in quartz cells, then formic acid (2 μl) was added to the solution. The solution was chromatographed using a PD 10 column (Pharmacia, Uppsala, Sweden) to remove the excess of **2**. The mixture was centrifuged (10 min, 5000 rpm) and 10 μl (100 pmol μl^{-1}) of the supernatant were injected to acquire ESI mass spectra.

Electrospray ionization mass spectrometry

ESI mass spectra were acquired on a VG Trio-2000 mass spectrometer (Fisons/VG, Manchester, UK) with an atmospheric pressure electrospray ion source and an interface employing a 75 μm i.d. fused-silica capillary inlet. The masses were analyzed with 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–formic acid (49.5:49.5:1, v/v/v) or 5 mM ammonium acetate (pH 6.0, adjusted with acetic acid). The flow-rate was 5 μl min^{-1}. Sample injection was carried out by means of Rheodyne Model 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 collisional 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 counter electrode voltage were optimized at 2.7 and 0.6 kV, respectively. The temperature of the capillary was set at 64 °C. The mass scale had been calibrated before, and then intermittently during the sequence of analyses using horse heart myoglobin (M_r 16951.5) (Sigma Chemical, Poole, Dorset, UK). Instrument control, data acquisition and some preliminary post-run processing used the VG LabBase software supplied with the instrument. Presentation of the raw m/z data on a true molecular mass scale employed the transformation software of the MassLynx package supplied with the VG Trio-2000. Precise molecular masses were determined using the maximum-entropy software (MaxEnt) incorporated in the VG MassLynx software package. Before the injection of the CaM–drug complexes, the capillary was washed with formic acid and then rinsed for 12 h with the carrier solvent (5 mM ammonium acetate) in order to remove traces of acid. All masses given in this paper are average molecular masses.

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

RESULTS AND DISCUSSION

The ESI mass spectra of the complex between CaM (M_r 16627 Da) and **1** were carried out in H_2O–CH_3OH (94:6, v/v) containing 5 mM ammonium acetate and 1 mM $CaCl_2$ (pH 6.0, adjusted with acetic acid); methanol was added to ensure complete dissolution of **1**. Under these circumstances, three series of peaks were detected corresponding to CaM[Ca]$_n$ ($n = 4$, 6, 9 and 10), CaM[Ca]$_n$–**1** ($n = 4$, 6 and 9) and CaM[Ca]$_n$–(**1**)$_2$ ($n = 4$, 6 or 10) (Fig. 1, Table 1).

We should emphasize one of the main problems with non-covalent studies by ESIMS. Under ideal experimental conditions (low salt concentration, aqueous–organic solvent, etc.), the ESIMS technique permits the measurement of masses with a precision of 0.01%.[21] The accuracy of our measurements is in the range 0.01–0.05% and reveals a general problem with complexation experiments. Preserving solution associations before transfer into the gas phase requires the use of aqueous media. High concentrations of salt (buffer and complexation agent) are also used. Hence under these experimental conditions we observe an increasing background noise and a broadening of signals (Fig. 1, inset). To

Table 1. Masses of the various CaM complexes[a]

Type	Complex	Measured mass (Da)	Calculated mass (Da)
CaM–(Ca)$_n$	CaM–(Ca)$_4$	16784 (14)	16779
	CaM–(Ca)$_6$	16860 (70)	16855
	CaM–(Ca)$_9$	16960 (65)	16969
	CaM–(Ca)$_{10}$	17010 (5)	17007
CaM–(Ca)$_n$–**1**	CaM–(Ca)$_4$–**1**	17046 (79)	17036
	CaM–(Ca)$_6$–**1**	17115 (100)	17112
	CaM–(Ca)$_9$–**1**	17230 (24)	17226
CaM–(Ca)$_n$–(**1**)$_2$	CaM–(Ca)$_4$–(**1**)$_2$	17294 (28)	17293
	CaM–(Ca)$_6$–(**1**)$_2$	17376 (38)	17369
	CaM–(Ca)$_{10}$–(**1**)$_2$	17532 (14)	17521
CaM–(Ca)$_n$	CaM–(Ca)$_4$	16779 (16)	16779
	CaM–(Ca)$_6$	16852 (58)	16855
	CaM–(Ca)$_{10}$	17012 (44)	17007
CaM–(Ca)$_n$–**2**	CaM–(Ca)$_4$–**2**	17106 (21)	17104
	CaM–(Ca)$_6$–**2**	17183 (100)	17180
	CaM–(Ca)$_{10}$–**2**	17330 (23)	17332
CaM–(Ca)$_n$–(**2**)$_2$	CaM–(Ca)$_4$–(**2**)$_2$	17420 (17)	17429
	CaM–(Ca)$_6$–(**2**)$_2$	17500 (20)	17505

[a] Measured and calculated peak masses obtained in the ESIMS study of complexes between CaM–**1** and CaM–**2**. The raw data were analyzed by the MaxEnt software at high resolution (1 Da per channel) over a narrow mass range in order to provide optimal resolution of the mixture. Relative intensities are given in parentheses.

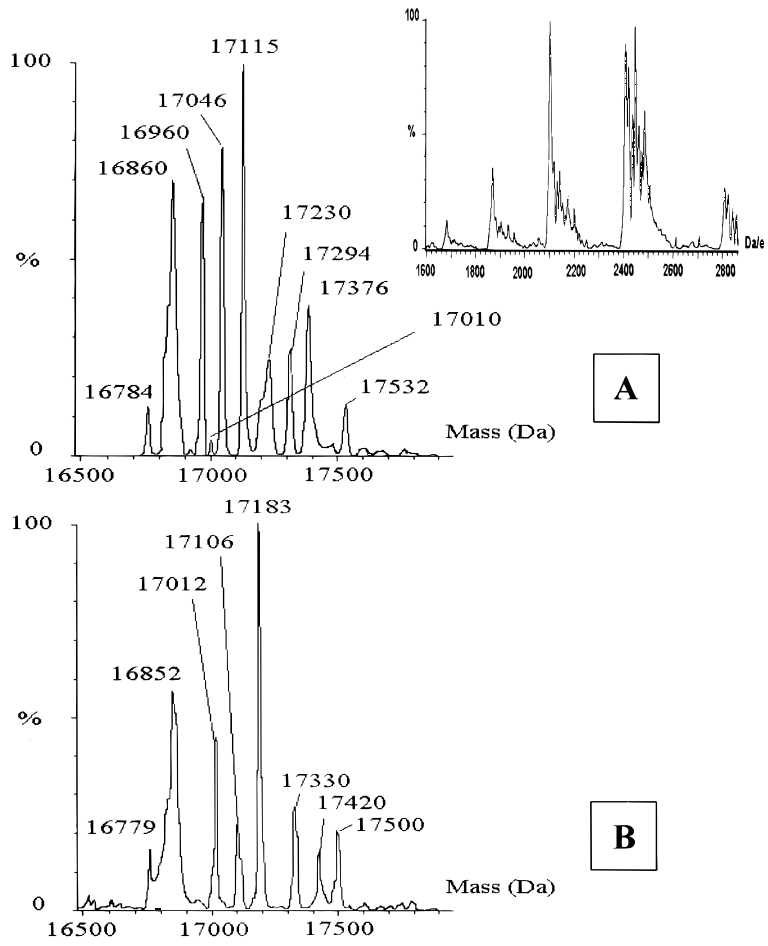

Figure 1. Mass spectra produced by the MaxEnt software processing of the raw electrospray data registered in ammonium acetate (5 mM, pH 6.0). (A) Non-covalent complexes of CaM[Ca]$_n$–**1** mixtures; (B) non-covalent complexes of CaM[Ca]$_n$–**2** mixtures (see Table 1). Inset: Raw data for the CaM[Ca]$_n$–**1** mixture.

avoid this phenomenon, an increase in the sampling cone voltage and a higher temperature are recommended,[22,23] but low non-covalent interactions can be lost.

Calcified CaM seems capable of accommodating up to two molecules of **1**. However, it must be noted that this stoichiometry could be underestimated owing to the weakness of the interaction between the protein and the drug. Indeed, a slight increase in the internal energy sufficient to induce a loss of one or several molecules of ligand may occur either during the desolvation process or by collision with residual gas. Therefore, we modified **1** by introducing a photoactivatable group, so it became possible to link together covalently the ligand and the protein by UV irradiation. Then, the comparison of the mass spectra registered before and after irradiation may allow the number of non-covalent interactions broken in the ESI process to be determined. Consequently, we synthesized **2**, in which a (diazomethyl)carbonyl group has been introduced. Since (diazomethyl)carbonyl compounds generate carbenes on exposure to UV radiation (Fig. 2), they have been used successfully for the photoaffinity labeling of several functional proteins.[24–26] These carbenes are more reactive species than, for example, related nitrenes.[24] Their very short lifetime involves reaction with very close neighbors, so free carbenes in solution react principally with their solvent cage. The use of a carbene and purified protein available in large amounts strongly minimized the non-specific labeling.[27]

As shown by microcalorimetry,[15] **2** binds CaM with an affinity similar to that observed for **1**. Moreover, the ESI mass spectra of CaM–**1** and CaM–**2** (Fig. 1, Table 1) are comparable. The only difference is the absence in CaM–**2** of the complex containing two organic ligands and nine or ten Ca^{2+} as observed for CaM[Ca]–**1** (Table 1).

As reported previously,[28] CaM is capable of accommodating up to 9–10 Ca^{2+} distributed between one main and two auxiliary classes of sites occupied by four, two and three (or four according to the experiment) Ca^{2+}. It must be noted that the binding of **1** and **2** to CaM is calcium dependent since no complex is detected in ESIMS with the apoprotein. Among the series of complexes containing the organic compounds, the more abundant species correspond to CaM[Ca]$_6$–**1**, CaM[Ca]$_6$–(**1**)$_2$ and CaM[Ca]$_6$–**2**. This is new evidence for the importance of the auxiliary calcium binding sites in calmodulin and their probable involvement in the regulation of interactions between the protein and its targets.

It must be noted that acidification of the solution containing the complexes with formic acid disrupts the non-covalent interactions giving unmodified CaM (data not shown).

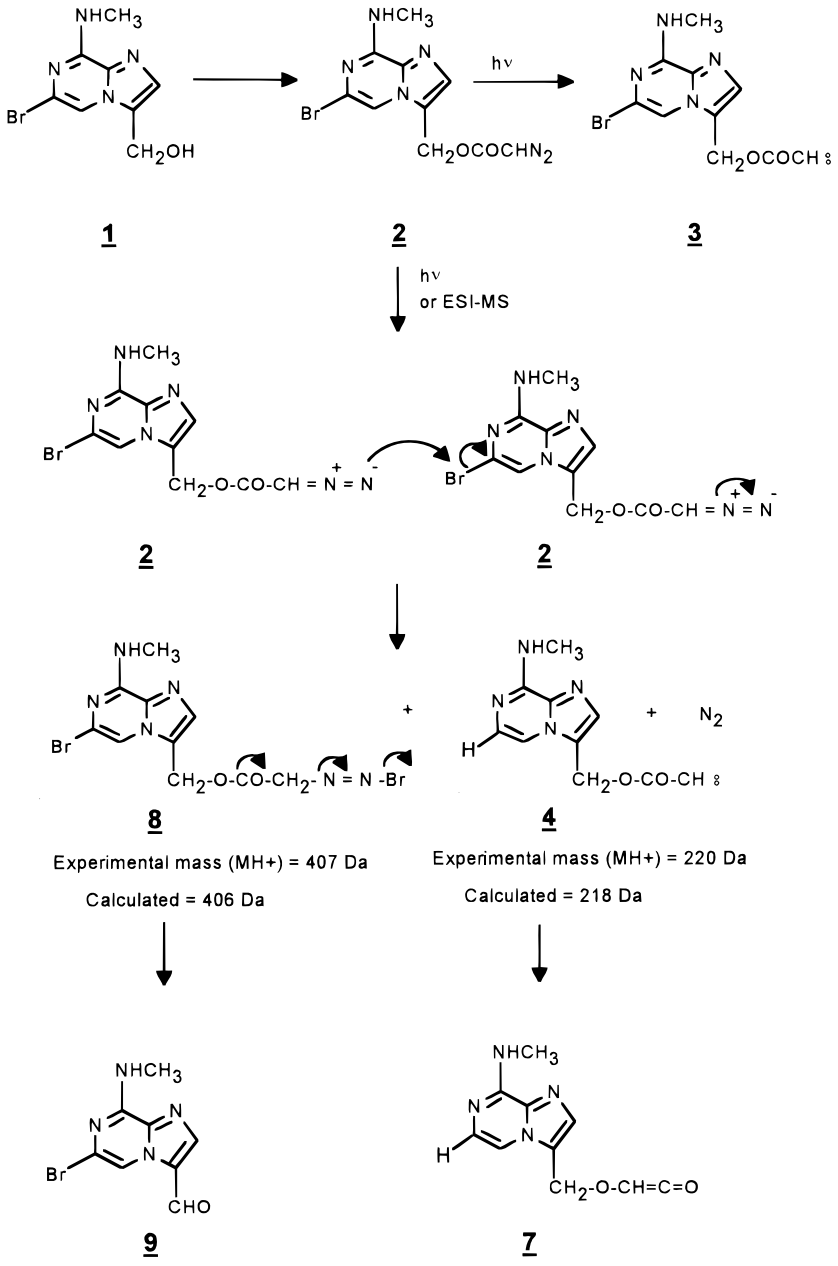

Figure 2. Molecular mechanism occurring during irradiation of the photoactivatable compound. Calculated masses are average molecular masses.

We then photo-labeled CaM with the (diazomethyl)-carbonyl derivative **2**. For this purpose, a solution of protein and **2** in methanol–water (94:6, v/v) containing 1 mM $CaCl_2$ and 5 mM ammonium acetate (pH 6.0) was irradiated at 254 nm for 30 min. After the irradiation, formic acid (2 µl) was added and the ESI mass spectrum was recorded in the positive-ion mode using acetonitrile–water–formic acid (49.5:49.5:1, v/v/v) as mobile phase. Under these conditions, the non-covalent bonds between Ca^{2+} and CaM are broken and only the molecules of **2** covalently bound to CaM remain. The raw spectrum of the mixture after irradiation is very different from that of CaM, showing the existence of three different species in solution (Fig. 3). This is confirmed by the MaxEnt analysis (Fig. 3). The major species with a mass of 16 641 Da corresponds to CaM (M_r 16 627 Da). It must be noted that during the photochemical labeling, a modification of the protein occurs since its molecular mass changes by about 14 Da (16 627 → 16 641 Da). We irradiated CaM alone and in the presence of the non-photoactivatable **1** using the same conditions as for **2**. We observed an identical mass increase (data not shown). This is certainly due to a photo-induced oxidation occurring at a methionine side-chain (—S—CH_3 → —SO—CH_3).

The two other peaks (17 710 and 17 812 Da) present in the spectrum (Fig. 3) correspond to the binding of four unmodified carbenes **3** (compound **5**, Fig. 4) and five carbenes having lost bromine, **4** (compound **6**, Fig. 4). It may be assumed that under UV irradiation **2** gives, in addition to the attempted carbene **3**, a species **4** (Fig. 4) without bromine. The mechanism of the dehalogenation could be a homolytic process. However, it must be observed that **1** is very stable when irradiated.

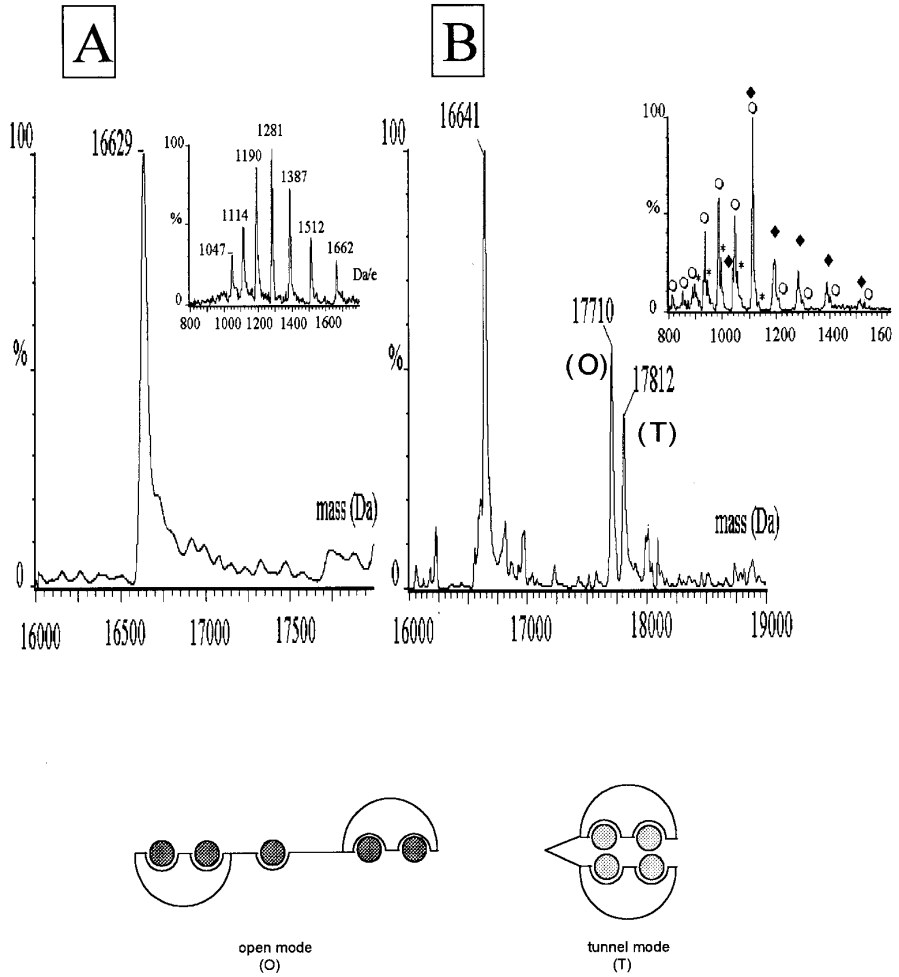

Figure 3. Mass spectra produced by MaxEnt software processing of the raw electrospray data registered in acetonitrile–water–formic acid (49.5 : 49.5 : 1, v/v/v) of CaM–**2** complexes before (A) and after irradiation (B). Insets: positive-ion-ESIMS (raw data); in (B) ◆, ○ and ✶ correspond to the peaks of CaM alone, CaM–**4** complex and CaM–**3** complex, respectively. In the open mode (O), grey circles correspond to the five debrominated carbenes (**4**) and in the tunnel mode (T), grey circles correspond to the four carbenes (**3**).

On the basis of chemical structures, it is difficult to understand this marked difference in the reactivity. More probably, the bromine abstraction occurs through a bimolecular process. Two molecules of **2** react with each other to give **4** and **8**. In a subsequent step, this latter derivative could decompose to give the aldehyde **9** (Fig. 2).

This mechanism was confirmed by studying the behavior of **1** and **2** alone by ESIMS. Compound **1** gives a single peak at 257 Da corresponding to the expected mass for the pure product (data not shown). In contrast, **2** decomposes into three compounds A, B and C with masses of 220, 253 and 407 Da, respectively. A (220 Da) corresponds roughly to the mass of the carbene without bromine (**4**). It is improbable that such a species could be detected by ESIMS owing to its high reactivity. Most likely, it rearranges to a ketene, **7** (Wolff rearrangement)[24] (Fig. 2). C (407 Da) corresponds to **8** and B (253 Da) could be the aldehyde **9** derived from **8** (Fig. 2).

It may be assumed that under UV irradiation and in presence of CaM, **2** gives the carbene **3**, which leads to **5** (Fig. 4). In addition to this reaction, **2** attacks a second molecule to give **4** (and **8**) as in ESIMS (Fig. 2). Then, CaM reacts with **4** to give **6** (Fig. 4). However, this figure cannot explain the observed results during the photo-labeling of CaM. If we assume that **3** and **4** are formed concomitantly, these two carbenes react very rapidly with *inter alia* nucleophilic groups (ROH, RSH) and double and single bonds, so we should observe mixed adducts containing both **3** and **4**. In contrast, if **4** appears a short time after **3**, we should detect adducts between CaM and the latter species only, **3** immediately reacting on all the potential sites and thus preventing the binding of **4**.

A logical explanation for the presence of two separate covalent complexes lies in the binding modes of CaM. The x-ray structure of this protein reveals two globular domains separated by a long solvent-exposed helix.[29,30] When CaM binds peptides or drugs, it undergoes a large conformational change resulting in a compact globular form.[31,32] The two lobes come together as a jaw to form a hydrophobic tunnel which wraps around the ligand (Fig. 3). In addition to this 'tunnel' mode, each lobe has the potential to interact with distinct targets, which could explain the evidence for the 2 : 1 stoichiometry of peptides binding to CaM ('open' mode, Fig. 3). It may be assumed that CaM can bind **2** in the open (O) and in the tunnel (T) mode. When the solution of the complex is irradiated, the carbene **3** reacts very

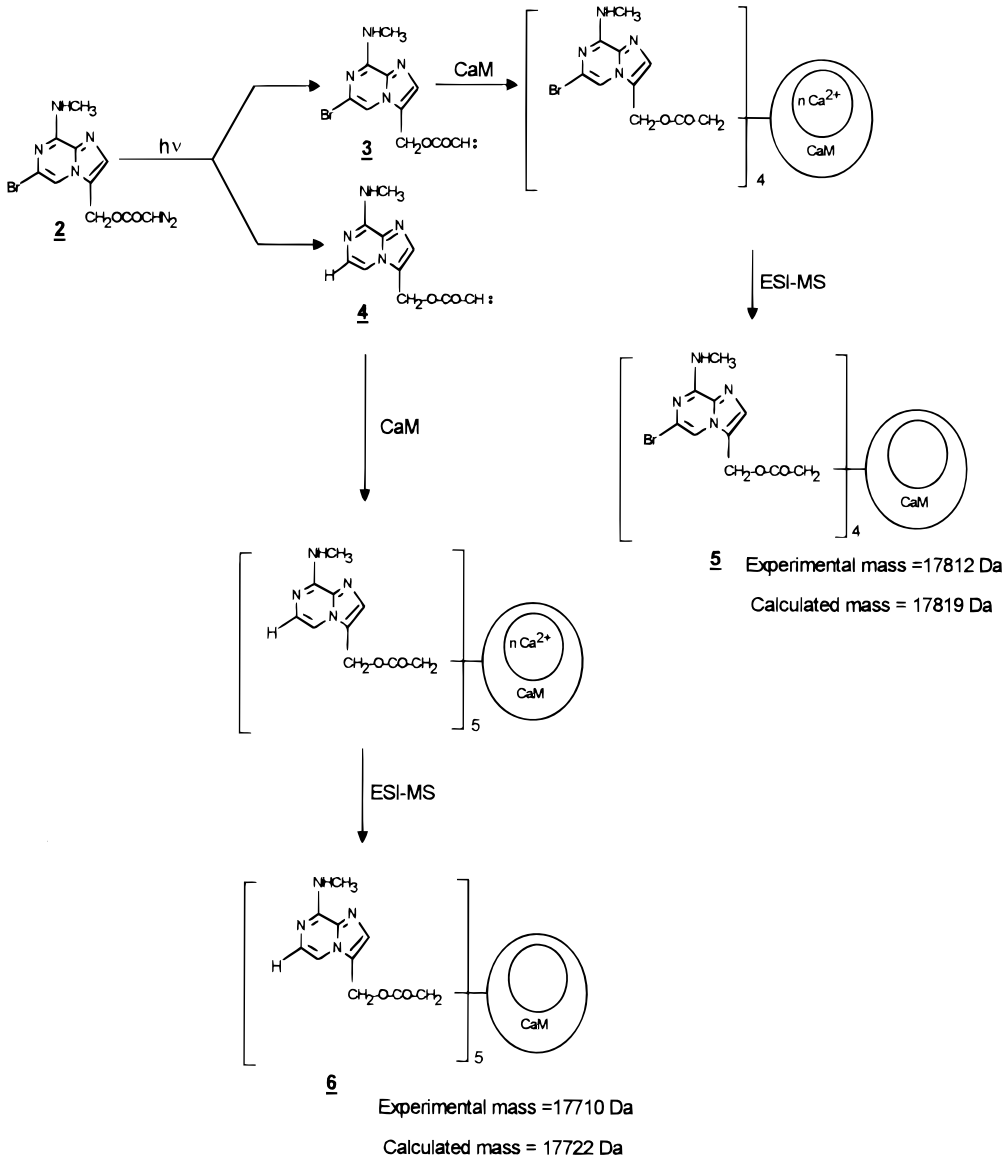

Figure 4. Photogeneration scheme of the two complexes detected by ESIMS: CaM–carbenes with (**5**) or without (**6**) bromine.

rapidly with the protein to give the T and the O states with four and five ligands, respectively (Fig. 3). In T, these ligands are buried within the protein and completely accessible in the O state. Under these circumstances, the excess of **3** in the surrounding solution can react with O only to remove the bromine according to the mechanism shown in Fig. 2.

From the results reported above, it appears that the stoichiometries of the non-covalent and covalent forms of the drug–protein complex are different. In the first case, two species are detected, corresponding to the binding of one and two organic molecules; it must be noted that the 1:1 adduct is largely preponderant. In the second case, we still observe two species but having four and five ligands covalently linked to the protein. At this time, it may be questioned whether the right number of drug molecules bound to CaM is that determined from the non-covalent adduct or that measured from the photoactivatable derivative after irradiation. Recently, Cook *et al.*[33] and Vandonselaar *et al.*[34] independently determined the crystal structure of CaM bound to trifluoperazine (TFP). Although the two structures appear overall similar, that of Cook *et al.* contains only one TFP and that of Vandonselaar *et al.* five. This difference must be related to the conditions of crystallization used: the ratio TFP to CaM was 4.5:1 in Cook *et al.*'s work and 35:1 in Vandonselaar *et al.*'s. It is possible that additional TFP molecules may bind non-specifically with very low occupancy at a high molar ratio of TFP to CaM. Such a phenomenon may occur in the study reported here. As we use a large excess of organic compound (about 10-fold), it is highly probable that five molecules bind to CaM in solution and during the volatilization process the non-specific links are lost owing to the increase in internal energy.

CONCLUSION

The usefulness of ESIMS to study non-covalent interactions in biological systems is well established. Numerous studies have been devoted to such systems, but they

are still a subject of considerable debate. As discussed above, it was important to establish the limit of stability beyond which it is not possible to detect the complexes. Indeed, such a limit must exist since during the transfer of ions from solution to the gas phase, the species are subjected to an increase in temperature, to strong electric fields and to collisions with residual molecules of gas.

In this work, we first compared the spectra of the two non-covalent complexes, CaM–(Ca^{2+})–**1** and CaM–(Ca^{2+})–**2**, in order to verify that the two organic derivatives behave in the same manner towards the protein. Then, CaM–**2** was irradiated and ESIMS data were collected. The observed difference in the stoichiometries of the covalent and non-covalent forms of the complexes clearly shows that a substantial number of interactions are lost during the ESI droplet desolvation process.

The difference in stoichiometry between the covalent and non-covalent forms of the complexes (two sites visualized instead of four or five) may be due to the existence of two classes of binding domains in CaM. The first class (two sites) gives complexes sufficiently stable ($K_d \approx 10^{-3}$ l mol^{-1}) to be detected, whereas the species built up from the second class (two or three sites) are too labile to withstand the desorption process.

In this work, we have confirmed that it is possible to visualize low-affinity complexes (10^{-3} l mol^{-1}). We have also shown that, it is possible to discriminate several affinity classes by ESIMS and that this will be useful for modeling other biophysical data.

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