Published in final edited form as:

J Proteome Res. 2013 April 5; 12(4): 1668–1677. doi:10.1021/pr301004w.

MALDI/Post Ionization-Ion Mobility Mass Spectrometry of Noncovalent Complexes of Dopamine Receptors' Epitopes

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Abstract

Protein domains involved in receptor heteromer formation are disordered and rich in the amino acids necessary for the formation of noncovalent complexes (NCX). We present mass spectral NCX data from proteins and protein receptors' epitopes obtained by combining ion mobility (IM) and MALDI. Our main focus are NCX involved in heteromer formation occurring between epitopes of the Dopamine D₂ (D₂R) and Adenosine A_{2A} receptors (A_{2A}R) as well as D₂R and a nicotinic (NR) receptors' subunit. The IM data yield information on the gas phase conformation of the singly charged NCX which are observed either directly from MALDI or as co-desorbed neutrals which are subsequently post-ionized by a time-delayed excimer laser pulse directed onto a portion of the neutral plume created by the MALDI desorption laser. Imaging mass spectrometry of the matrix/epitope dried droplet surface shows that the acidic and basic epitopes and their NCX are found to be spatially collocated within regions as small as 25×50 microns. Subtle differences in the relative abundance of protonated and cationized NCX and epitopes are measured in spatial regions near the sodium rich outer border of the droplet.

Keywords

Noncovalent interactions; ion-mobility; disordered proteins; dopamine receptor; heteromers

Introduction

The study of biomolecular interactions which form noncovalent complexes (NCX) is essential for understanding how structure translates into function (1-18). Heteromer formation is used by G protein-coupled receptors to diversify their biochemical characteristics with respect to those of individual receptors (19). Finding and testing the epitopes involved in receptor heteromerization (11-18), especially the dopamine D₂ (D₂R)-Adenosine A_{2A} (A_{2A}R) receptors [Figure 1a], has been a primary focus of our work on noncovalent bonding. The phosphorylating enzymes involved in heteromerization (14,18)

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give specificity to the interaction between the receptors' epitopes. We have identified such complementary epitopes and demonstrated that they form NCX using mass spectrometry (11-18), pull down techniques (13), patch clamp recording (17), mutations and sequential FRET and BRET (13,18). Bioinformatics analysis by Agnati et al. has shown that these epitopes are found in highly disordered domains (19), which explains the many possibilities for conformational modification before and during the formation of the receptor complex.

Such proteins, or their segments that lack three-dimensional structure, are called "Disordered regions" as described in Dyson and Wright's excellent review [20] and by Romero P et al.(21). These proteins are most often highly conserved between species in both composition and sequence, as is the case for the A_{2A}R and D₂R (13). Moreover, contrary to the traditional view that a protein's conformation or three-dimensional structure determines function, it is the disordered regions that are most important for the formation of NCXs. These regions [2,9,19,22] are rich in disorder promoting amino acids (A, R, G, Q, S, P, E and K), lack order-promoting residues (W, C, F, I, Y, V, L and N), and possess an overall high net charge and low hydrophobicity (Figure 1b). Thus epitopes involved in the initiation of receptor heteromerisation do it through salt bridge formation primarily driven by an electrostatic and not a hydrophobic interaction [18-20]. Upon binding to their biological targets, the disordered segments of a protein usually fold, thus acquiring a conformation that the disordered protein lacked. The disorder to order transition, during binding of disordered regions, makes the interaction reversible, yet highly specific [21].

Modern Mass spectrometry has made it possible to extensively study noncovalent complexes (NCX) (2-18,22-24). Since the NCX formation takes place in the liquid phase, it was initially thought that Electro-spray ionization would be the method of choice for these studies (4,22), as it provides highly charged large complexes, which can then be measured at reasonably low *m/z*. Nevertheless, we have previously demonstrated that useful information from singly charged NCX could be obtained with MALDI TOF instruments (6,9) if matrices with pH>4.5 are used. Advantages of MALDI for detecting NCX include the predominance of singly charged ions, the use of aqueous solution for sample preparation and biocompatible matrices such as DHB or DHA, as well as MALDI's tolerance to high salt concentrations, which preclude the use of ESI. Since most biological processes take place in regions of high ionic strength this is a very complementary distinction between the two techniques.

We originally combined ion mobility separation and MALDI for the study of peptide NCXs which form between two adjacent Arg on one peptide and an adjacent Glu/Asp sequence on the other(10,11) and demonstrated salt bridges formation between the epitopes of the involved receptors (12,14). Our subsequent studies showed that phosphorylated epitopes form even more stable NCX when paired with basic epitopes regions containing either two adjacent Arg (14, 15,25-27) or quaternary amines (2,28).

In this work, MALDI-IM-*o*TOFMS (MALDI-Ion Mobility-orthogonal time of flight mass spectrometry) [Figure 2] is used to augment the study of such noncovalent bonds and their importance in protein complex formation as exemplified by receptors' heteromerization. IM is sensitive to differences in peptide and protein gas phase conformations and completely

separates charge states which clarify the MALDI spectra of singly charged ions. The ions' drift time in the electrically biased helium filled IM cell are determined by their gas phase densities. Small compact ions drift more quickly than open ion structures of the same mass which are slowed by more frequent collisions with the low pressure helium gas as they traverse the ion mobility cell. Thus differences in the gas phase ion structure can be inferred and inter-compared within and between singly and multiply charged MALDI ions. For example, one of our earlier works with MALDI-IM-o-TOF MS first defined its bio-analytical utility by separating ions from mixtures of lipids, peptides and nucleotides according to chemical type along well-defined chemically distinct “trend lines” in plots of ion drift time vs. m/z (29). Furthermore, within a particular class, subtle differences between structures can also be distinguished by IM-MS, e.g. lipids can be distinguished by 1) degrees of saturation of the fatty acid tail, 2) composition of their head group, and 3) cationization (29,30).

Our present study uses advanced MALDI-IM-oTOFMS instrumentation and software (31) to emphasize the importance of the electrostatic interaction between the D₂R third intracellular loop (IL3) and the A_{2A}R carboxyl terminus epitopes, as indeed the first and necessary step in receptor heteromer formation. The epitopes chosen for this work belong to the receptor domains involved in NCX formation: D₂R (VLRRRRKRVN); NRNRVEAARR, both located in the long isoform of the D₂R; A_{2A}R (SAQEpSQGNT); and β 2 Nicotinic (pSEDDDDQpSVSED) receptor (NR) subunit, which play important roles in brain neurophysiology and the pathology of addiction. We also measure NCX formation between the basic epitopes from the D₂R long isoform and the A_{2A}R and NR phosphorylated acidic epitopes as well as Calmodulin. Moreover, for the first time within the ion mobility, *in-source* vacuum ultraviolet (VUV) post-ionization and photo-fragmentation [31] of neutral Calmodulin and its NCX with D₂R epitopes are measured. We conclusively resolve a longstanding question within the MALDI NCX research community by using imaging IM-MS to prove the co-location of free epitopes and their NCX within dried droplets matrix preparations at a 50 micron pixel spatial resolution. MALDI signals from both protonated and alkali adducted ions are simultaneously produced from each pixel and can be used to estimate the effect of ionic strength on NCX formation.

Material and Methods

Peptides—The following receptor epitopes (peptides) were synthesized at the Johns Hopkins School of Medicine Synthesis Core Facility and their structure and weights are given as follows: VLRRRRKRVN (ppt 1, from D₂R; 1351.9 amu), SAQEpSQGNT (ppt 2, from A_{2A}R; 1000.4 amu), NRNRVEAARR (ppt 3, from D₂R; 1282.7 amu), pSEDDDDQpSVSED (ppt 4, from nicotinic R; 1384.4 amu), RRRKRVNTKRSSR (ppt5, which is also the interactive D₂R epitope 1855.13) CaM (16997 amu) was purchased from Millipore. All peptides were dissolved in water. A “4700 peptide standard” commercially available from AB-SCIEX made of seven non-phosphorylated peptides was used both for m/z and Ion mobility trend line calibration of non-phosphorylated peptides.

Sample prep—Equimolar solutions (10 picomoles/ μL) of acidic and basic peptides were prepared and analyzed. The matrices used were 2,4-dihydroxyacetophenone (DHA) or 2,4,6-trihydroxyacetophenone (THA). To 0.3 μL peptides mixture was added 0.3 μL matrix on the sample plate. Since DHA matrix rapidly sublimates, mass spectra should be acquired within an hour of introducing the sample in the instrument. The less volatile DHB was also used as matrix which not only gives results consistent with the DHA, but is found to be an excellent matrix to promote post-ionization of peptides and lipids [31].

MALDI-Ion Mobility-oTOF mass spectrometry (MALDI-IM-oTOFMS) using an ion mobility drift cell

Figure 2 shows a MALDI-Ion Mobility-oTOF mass spectrometer (MALDI-IM-oTOFMS) incorporating an integral ion mobility technique, which measures the drift velocity of all pulse laser desorbed ions through helium gas. The usual dried droplet matrix preparations on a standard MALDI plate are located within a 2 Torr helium filled source region. The desorbed ions are drawn into the Ion Mobility (IM) cell by a voltage between the sample and the entrance to the helium filled IM cell. An ion's drift velocity through the helium gas is proportional to the ratio of the ion's charge/volume. Separation of ions with the same mass but different surface areas is thus possible. After a MALDI desorption pulse, small or dense ions quickly traverse and exit the IM cell and enter the mass spectrometer where the ion's m/z is measured when a pulsed voltage deflects the nearly parallel ion beam into a reflectron time of flight mass spectrometer. The ion's m/z and its drift time (which is measured from the moment of desorption until the application of the orthogonal extraction pulse) are both stored in computer memory. Larger or less dense ions elute from the cell over a longer time period (a millisecond for 10 kDa ions). Ion drift times are typically under one millisecond for m/z 5000 yet the time of flight in the mass spectrometer is only a few tens of microseconds. Thus after each UV laser desorption pulse multiple mass spectra are acquired so that the drift time and m/z of each ion desorbed from each MALDI desorption pulse can be sorted and presented either in the form of a 2D color intensity plot or a 3D color perspective plot. In such displays of ion intensity, m/z , and drift time, similar molecules appear separated along familial trend lines (e.g., lipids, peptides, and nucleotides are separated from each other by their shape) (29,30). The one dimensional mass spectrum (on top of the color intensity plot) is numerically derived from the 3D data by adding all ion signals at each m/z irrespective of their mobility; this summed spectrum is similar to what is seen in conventional MALDI-MS. Likewise, the corresponding 1D ion mobility spectra are derived by summing all ions as a function of their mobility drift times irrespective of their m/z . In some cases, these derived spectra are obtained by constructing a window around a region of interest in the mobility IM- m/z 3D space. For example, if the IM- m/z region around a protein MH^+ is selected, the derived mass spectrum is then uncontaminated by the “chemical noise” of multiply charged ions. A fourth data dimension is obtained by adding the VUV post-ionization of desorbed neutrals and appears in the color intensity plots as a second set of trend lines separated from the MALDI by the time delay between the desorption laser and the VUV post-ionization laser pulses. These data and their acquisition are further described below.

Post-ionization—Figure 2 shows how neutral molecules which are co-desorbed during MALDI can be turned into useful analyte ions with a second laser (F₂ laser 7.8 eV) which is aligned parallel to the MALDI sample surface and is focused into a volume of a few hundred cubic microns at a distance of 0.5 mm above the sample. The neutral molecules which are desorbed with significant velocity quickly stagnate within this volume by collisions with helium and can be post-ionized by the VUV laser either by direct photo-ionization or photo-fragmentation. The experimental sequence is as follows: First the pulsed YLF (349 nm) laser simultaneously desorbs MALDI ions and neutrals from the sample. After waiting four hundred microseconds, all the direct MALDI ions will have entered the IM cell under the influence of the static electric fields between the sample and the cell entrance. At this time a 50 × 500 micron focused VUV (157 nm) laser pulse impinges the desorbed neutrals which have been ejected from the surface into a volume a few hundred microns above the surface. The initially fast neutrals lose translational energy, stagnate, begin to diffuse, and are slowly transported toward the IM cell by a weak Helium gas flow directed down the cell bore. When the VUV creates ions from a portion of these neutrals they experience the electrostatic extraction force between the sample and the entrance of the IM cell and immediately begin drifting into the IM cell. Thus the plots of drift time vs. m/z will contain a set of two trend lines (one from the MALDI and one from the post-ionization) which are uniformly offset by the 400 microsecond delay between desorption and post-ionization laser pulses. We estimate from molecular dynamics simulations (data not shown) that CaM and CaM-D₂ NCX collisionally cool within about 20 microseconds to produce a stagnant 0.5 mm diameter volume of neutral molecules just above the surface, assuming an initial velocity of 500 m/sec for the proteins. Thus, at present, the line focused VUV laser probably intersects no more than about 1-2% of the protein neutral plume. By varying the time delay of the post-ionization laser pulse relative to the MALDI pulse over the times 0.1 to 1 msec (data not shown), we determine that the neutral peptide and proteins persist above the surface with an estimated decay constant of around 500 microseconds before they are carried away from the source region by the helium gas flow and by diffusion.

Also shown in figure 2 but not used in the present study, is a second post-ionization laser (also a YLF) which can be used to create analyte ions from laser desorbed neutrals by matrix declustering (31,32). This gas phase MALDI declustering within an IM cell is particularly powerful resulting in almost pure MH⁺ lipid and peptide spectra both from tissue and from pure standards (31).

Instrument—Figure 2 shows a schematic of a MALDI- Ion mobility (IM)-oTOFMS imaging spectrometer (Ionwerks Inc. Houston TX) which was used in positive ion mode. The MALDI desorption laser is a YLF (349 nm Spectra Physics) focused within a 20 micron spot onto the MALDI target. The post-ionization laser is an F₂ VUV light source (157 nm (7.8 eV) GAM corporation) which is focused parallel to the surface and into an approximately 0.1×0.5 mm cross-section located approximately 0.5 mm above the sample surface. The firing of the post-ionization laser is time delayed by 400 microseconds after the MALDI desorption. The 14 cm long IM cell is filled with 2 Torr He and achieves a mobility resolution of 30 (FWHM) and a mass resolutions of 3000 in both MALDI and post-ionization data using C₆₀ and angiotensin standards. Protein ions (20 kDa) traverse this cell

in less than 4 milli-seconds which allows desorption laser pulse rates of 250 Hz. MALDI-IM-oTOFMS. This separation provides three analysis of differences in the gas phase molecular conformation of biological families such as lipids, peptides, and nucleotides, in which isobars are separated from each other by their IM drift times [29] and a fourth dimension in which direct ions and postionized neutrals [31] are distinguished. Although singly charged ions are predominant in MALDI, the complete resolution of singly and multiply charged ions is a particularly useful feature of integral ion mobility since the chemical noise background is now transformed into useful multiple charge state ions purified into trend lines according to their m/z .

Surface molecular Imaging with integral Ion Mobility

Molecular Imaging of the spatial location of pSEDDDDQpSVSED (ppt4) + NRRRVEAARR (ppt3) on the dried droplet matrix/analyte surface were acquired using new software and hardware capabilities which enables mass spectra of all ions at two or more distinct ion mobility drift times. The instrumental software has the capability to offset the start of mass spectral data acquisition to a specific drift time, and by only allowing for one TOF extraction cycle, a single narrow IM drift time centered on the NCX can be optimally sampled by every other laser shot at high repetition rates. A second set of mass spectra is correlated with a shorter IM drift time which encompasses both the acidic and basic epitopes by moving the laser trigger to time align with this second IM drift region of interest. In this mode of operation, the laser trigger toggles between two timing states, in this particular instance separated by 200 micro-seconds.

After every laser pulse, each desorbed ion's mobility and m/z is recorded and each ion's mobility resolved molecular surface images are reconstructed. The 200Hz MALDI laser, is turned on for the entire 3mm x-axis line scan with the x-y stage moving at 50 micron/sec (or 25 micron/sec data not shown). Data is saved over the two IM filtered mass ranges every second (i.e., every 50 microns). At the end of the +x direction line scan, the stage increments in the y-direction one pixel (50 microns), and the stage movement direction is reversed and a second line scan proceeds in the -x direction. Each line scan completes in 1 minute (3000 micron/50micron/s = 60s) with 60 scans required in total for a 1 hr of imaging time. Signal is acquired from within each 50 micron pixel from a small area which is approximately 500 square microns (50 micron pixel $\times$ 10 micron laser FWHM width). Thus approximately 20% of the surface area of the dried droplet is irradiated during the acquisition of these images.

Results and Discussion

MALDI-IM-oTOFMS spectra shown in Fig. 3 are from sample mixtures containing the following pairs of peptides [VLRRRRKRVN (D_2R , ppt 1) + SAQEpSQGNT ($A_{2A}R$, ppt 2)] while data in figure 4 are from [NRRRVEAARR (D_2R , ppt3) + pSEDDDDQpSVSED (NR, ppt4)]. In the text, we report the molecular ions' m/z (MH^+) in amu and their drift times (DT) in micro-seconds (μs) using the shorthand (MH^+T). All trend line approximations are linear connections between MH^+ and M_2H^+ of either the acidic or the basic peptides. The linear fit serves not only to guide the eye but to illustrate the nearly 10% difference in isobaric separation between the acidic and basic peptides. This difference was confirmed (data not shown) by overlaying the trend line fit of the non-phosphorylated peptide standard

mixture, which falls on top of the basic epitope trend line. The basic peptide trend line is solid (blue) and the acidic peptide is dashed (red).

NCX between D₂R and A₂A_R epitopes—Figure 3a shows the peaks from VLRRRRKRVN (ppt1) 1352.9 amu: 410 μ s, SAQEpSQGNT (ppt2) 1001.4 amu: 320 μ s, and the NCX at 2353.3 amu: 534 μ s (Fig. 1a). Since the ppt1 contains 6 adjacent basic residues it can also form NCXs with two ppt2s at 3353.7 amu: 643 μ s and three ppt2s at 4452.7 amu: 736 μ s (not shown in the figure). The trend line for the NCXs falls between the trend lines of ppt1 MH⁺ (solid blue) and ppt2 MH⁺ (dashed red). The MH²⁺ trend line (dashed purple) has an even faster mobility than either of the singly charged peptides. It is interesting that the doubly charged ppt1, ppt2 and their NCXs are all on one trend line and do not show the conformational variations so evident in the singly charged peptides [Figures 3a and b].

Although the arginine-phosphate electrostatic interaction is noncovalent, it possesses a “covalent-like” stability enabling the NCX to withstand fragmentation by CID at energies similar to or larger than those which fragment covalent bonds in similar sized molecules [14-16,25-28]. Our previous studies proved that the gas phase phosphate-guanidinium interaction is so stable that the covalent bond between the phosphorylated serine's oxygen and the HPO₃'s phosphorus cleaves, so that the HPO₃ remained with the guanidinium containing peptide rather than dissociating (13-16,25-28). Herein we see that the ion mobility of singly charged ions proves that the phosphate group (H₃PO₄=98 amu) remains noncovalently bound to ppt1 as evidenced by the 1450.9 amu: 431 μ s peak in the MALDI-IM-oTOFMS spectra in Fig.3b. This peak is comparable in intensity to that of ppt2.

Some care is required in interpreting why ppt1 + H₃PO₄ is on the non-phosphorylated trend line along with basic ppt1 in figure 3b since phosphorylation almost always results in densification of singly charged gas phase peptide ions (33,34) which gives them faster drift times. Interpretation of these data starts with postulating an *in-source* NCX (ppt1+2ppt2) which upon *in-source* fragmentation generates either ppt1+H₃PO₄ or ppt1+2H₃PO₄. Both of phosphate adducted ppt1 fragments, along with ppt1, traverse the IM cell intact up to the interface. However, upon entering the interface region between the IM cell and the oTOFMS neutral loss of phosphate can occur by collision induced dissociation. In fig 3b two phosphates remain attached to ppt1 yielding a weak peak [ppt1 + 2(H₃PO₄) (1548.9 amu: 423 μ s)]. The stronger peak, however, occurs for the neutral loss of phosphate producing the ppt1 + H₃PO₄ fragment which appears at an IM drift time nearly identical to the parent (in figure 3b the two are connected with a horizontal dashed line). Additional support for this interpretation comes by connecting the IM centroids of ppt1 and ppt1 + 2(H₃PO₄) contours with a second inclined dashed line which goes through a weak shoulder peak at the lower drift time of the ppt1 + H₃PO₄ contour. It is important to understand that the only way in which gas phase ion configurations can be unambiguously assigned by ion mobility mass spectrometry, is to pre-select the source ion of interest with a mass filter prior to injecting the ion into the IM cell [35]. Fragmentation is an ever present possibility either within or after any type of IM device—whether differential or integral; however, with integral ion mobility one can tell where this fragmentation occurs.

By using an integral ion mobility instrument, such *in-source* and interface fragmentations are identified as seen in Figure 3b. These phosphates on ppt1 came from ppt2 showing yet again that the noncovalent bond between the basic peptide and the phosphate is stronger than the bond between the phosphate and the serine residues (14). On a side note, the *in-source* retention of the one and two phosphates by the basic ppt1 results in a densification of its gas phase structure similar to that observed previously for acid peptides [14,34,35]. This effect is also seen in higher mass clusters in Fig 3a where an additional H₃PO₄ is adducted to the NCX at 2451.3 amu:545 μ s (NCX+ H₃PO₄) [Figure 2a].

NCX of D2R and Nicotinic Receptor epitopes

Choosing epitopes with more than one potential binding site enables formation of a variety of NCX conformations which lead to increasing complexity of the gas phase IM spectra. We illustrate this point in Figure 4 with epitopes of the Nicotinic and D₂R receptors. The acidic peptide **pSEDDQpSVSED NR ppt4** (1283.7 amu:450 μ s) has a double phosphorylation, while the **NRRRVEAARR (D₂R),ppt3** (1385.4 amu:435 μ s) has two groups of adjacent arginine residues in separate regions of the peptide. Thus multiple configurations of the NCX between these two epitopes are possible in which one or more RR and acidic sites interact during NCX formation.

The data in Figure 4 shows an NCX peak at (2668.1 amu:655 μ s). The weak peak at 2570.1 amu:650 μ s is generated by the loss of one of the two phosphates from the NCX. As previously seen in Figure 3 (ppt1, ppt2), we again detect a peak showing the phosphate breaking from the acidic peptide, and remaining noncovalently bound to the basic epitope [Figure 4b] 1381.7 amu:470 μ s. Also seen in figure 4b, is the *in-source* loss of one phosphate from acidic **ppt4**, generating a fragment which although nearly at the same *m/z* as **ppt3** has slightly faster drift velocity.

We also detected multimers of three NCXs formed by the addition of two basic peptides to the acidic epitope at 3950.8 amu: 834 μ s and the addition of two acidic peptides to the basic epitope, at 4052.5 amu: 818 μ s. The drift time of the NCX containing two basic epitopes is greater than that of the NCX with two acidic epitopes. Phosphorylated epitopes were detected along trend lines but at shorter mobility drift times than the basic epitopes. The phosphate neutral loss peak appears on a nearly horizontal line to the left of the NCX. We also measure the MH²⁺ of the three NCXs at 1334.6 amu:375 μ s, 1975.9 amu:465 μ s and 2026.9:amu:468 μ s respectively.

The mobility data of figures 3 and 4 are consistent with our previous findings that epitope-epitope electrostatic interaction represents an important general mechanism underlying receptor-receptor communications [14,18]. In the case of the D₂R- A_{2A}R epitope interactions, the addition of calmodulin (CaM) to the mixture displaced the Adenosine A_{2A}R epitope and instead CaM formed stable NCX with the D₂R epitope (data not shown).

What happens when the basic D₂R epitope interacts with Calmodulin?

Calmodulin (CaM) and **VLRRRRKRNVN (ppt1)** and their NCX are shown in [Fig. 5a]: CaMH⁺, 16696.8 amu:2350 μ s, and the following CaM(ppt1)_x NCXs; CaM(ppt1) at 18047.7

amu:2419 μ s, CaM(ppt1)₂ at 19400.6 amu:2540 μ s, CaM(ppt1)₃ at 20752.5 amu:2620 μ s and CaM(ppt1)₄ at 22104.4 amu:2750 μ s. All these peaks are seen along the same trend line which is coincidentally the same protein trend line defined by the CaM and CaM₂ and their *in-source* fragments. Thus, the NCX are shown to have the same gas phase ion mobility cross-sections as an *in-source* protein fragment peptide of identical weight—the formation of the NCX creates a gas phase density which is identical to that of the protein (within an IM resolutions of 30).

However, the matrix adduct ions, show a very different gas phase ion structure compared to that of the NCX. The CaM(Matrix)_x⁺ drift times change very little relative to the CaMH⁺ even as successive matrix molecules adduct. This is seen by the nearly horizontal matrix adduct trend line. This means that the gas phase structures of CaMH⁺ and CaM(matrix)_x are of similar surface area. Thus the matrix molecules must be integrated into the internal tertiary structure of the protein so that the CaM[Matrix] is of greater density than the CaM. The protein and its matrix adducts have nearly the same charge to volume ratio (near identical drift times). A one percent difference in the drift time of CaM(Matrix)₃ relative to the protein of similar weight can be measured from the two different trend lines in figure 5b.

In Figure 5 a and b, the MH²⁺ ions are seen on their own trend line below the MH⁺. The higher charged multimers are also clearly delineated, and we observe trend lines of decreasing intensity down to MH⁵⁺. It is not surprising that so many gas phase NCX form with CaM, since the proteins contains 38 acidic and 10 aromatics residues, many of them adjacent (2,8).

Post-Ionization of neutral D₂R epitope (ppt5), Calmodulin and their NCX

At this point we demonstrate that a substantial portion of the protein-peptide NCX is desorbed as gas phase neutral complexes. Using DHB matrix, co-desorbed neutral epitopes, proteins and their neutral NCX complexes can be post-ionized and partially fragmented by firing a VUV laser into the co-desorbed neutral plume four hundred microseconds after MALDI ion desorption. These data are displayed in Figure 6 a and 6b in which the MALDI desorption MH⁺ are along the lower trend line while the singly charged post-ionized neutrals are along a trend line offset by 400 microseconds.

A longer epitope of the D₂R (ppt5) **RRRRKR**VNTKRSSR (1855.13) (which contains almost all the sequence of ppt1 and is from the same active site) was used to form neutral NCXs with CaM using a DHB matrix. In agreement with the MALDI data for CaM(ppt1)_x NCX (shown previously in Figure 5), the corresponding CaMH⁺ and CaMH⁺(ppt5)_{1,2} and 3 are seen in Figure 6a to align along the trend line connecting the in-source fragments and CaM with its dimer CaM₂. Also, the drift time invariance of the DHB matrix adducted CaM trend line is even more pronounced (slightly negative sloped trend line in Figure 6a and 6b) compared to the slightly positive sloping matrix adduct trend line of the heavier DHA adducts shown in figure 5.

The post-ionized neutral trend line shows intact ppt5 and CaM as well as their NCXs. Comparison of MALDI and Post-ionization is aided by the red boxes encompassing MALDI and neutral post-ionization regions of interest around ppt5 and around the CaM-ppt5 NCX.

Post-ionized ppt5 is one third the intensity of the ppt5 MALDI ions. In Figure 6b the intensity of the MALDI and Postionized CaM and CaM-ppt5 ions is approximately equal; however, the integrated intensity of the postionized CaM and CaM-ppt5 and their fragments are broadly distributed around the nominal masses. The Postionization intensities occur from 1-2% of the neutral plume which is ionized by the VUV laser.

A substantial number of photo fragment ions are seen all along the singly charged neutral post-ionized trend line and at masses below the intact ppt5. Although a few of these masses can be assigned to structural fragments created by VUV photo-fragmentation of the amide bonds in ppt5 (36,37) the preponderance of the smaller post-ionized neutrals cannot be assigned to ppt5 and must therefore originate from the in-source fragmentation of some of the CaM. In fact the singly charged neutral post-ionized trend line, when viewed from low mass to high mass, shows many low intensity ions at every mass, even though only ppt5, CaM, their NCXs and matrix are in the dried droplet mixture. Thus, upon comparing Figs. 6a and 6b, we see that the smaller ions are only in part created by direct fragmentation of ppt5. Mostly the small mass ions come also from photo-fragmentation of intact CaM or NCX or even larger dimers of the two. Alternatively some can occur through *in-source* fragmentation of neutrals during the MALDI desorption. An *in-source* fragmentation of these intact neutral protein and peptides could create smaller neutral molecules which are then further photo-fragmented and ionized by the VUV which also contributes to the plethora of peaks along the MH^+ trend line.

The *in-source* VUV photo-fragmentation and post-ionization of intact neutral protein and protein complexes is novel and potentially very useful since this clearly produces many large protein fragment ions which could be used in sequencing protein structure including structure of the protein-peptide NCX. In the future, such studies will benefit from an experimental configuration in which UV (eg. 349 nm) postionization is first used to form ions from desorbed neutrals by gas phase MALDI matrix declustering which is then followed by VUV fragmentation of selected ions within the middle region of the IM cell. Photofragments specifically along a separate trend line could then be specifically correlated with the ion mobility purified region around a chosen molecule (31). This method will provide on-demand photofragmentation of mobility separated peptides and NCXs.

Imaging of the spatial distributions of acidic, basic and noncovalent complexes on and within salty DHB dried droplet surfaces

In figures 7 and 8 to ease visualization we substituted the abbreviation **A** for acidic **ppt4** and **B** for basic **ppt3**. In Figure 7 (left panel) we show a laser microprobe image labeled (A+B) derived by summing all signals (MH^+ and adducts) within each 50 micron pixel related to **ppt3** and **ppt4** over m/z 1200-1500. In the right panel of Figure 7 is shown the simultaneously acquired **NCX** related signal ($NCXH^+$ and all NCX alkali adducts) within the m/z region from 2650 to 2750 ($NCXH^+$ and alkali adducts). The resulting image shows the variation of the combined ion intensity over the entire 3mm diameter dried droplet. The spatial co-location of the ions related to the acid and basic peptides with the NCX related ions and adducts is striking. The likelihood of the co-location of the NCX and its free constituent peptides has been debated for years. Our results unambiguously show co-

location of all signals in spatial regions at least as small as 10×50 microns (co-location was observed at 20×10 pixel-- data not shown). With these results from ppt3 and ppt4, we have demonstrated a method to determine if this co-location generally holds true in other cases of matrix assisted desorption of NCX.

Interesting subtleties between alkali, protonated peptides and NCX arise as we further derived images shown in Figure 8. From the same ion mobility imaging data we compare the pure protonated or cationized peptide ions (denoted AH or ANa for **ppt4**) and (BH or BNa for **ppt3**) and the protonated or cationized NCXH or NCXNa. The white number in the upper right corner of each panel denotes the total ions counted in the most intense 50 micron pixel of each image. The singly sodiated peptides and NCXNa are in a row below. We observe differences in the distribution of the Na and H adducted peptides. AH is most intense in the center of the droplet, and BH near the edges of the droplet. While both ANa and the BNa are found near the edges of the dried droplet, the BH tracks the intensity profile of ANa, namely more intensity in the middle than at the edge. BNa is almost uniformly spread across the droplet, and if anything is more intense near the edge than at the center. The NCXH, NCXNa, and AH and BH are very similar in spatial intensity distributions. The drying of the droplet, particularly with substantial sodium in the solutions is clearly a very complicated process with salting the basic peptide out of solution near the edges of the droplet as the droplet dries from outside to in. In the future, the IM imaging technique can be used to measure the effects of different cations on the adduct segregation and degree of co-location of adducted peptides and NCX.

Future Directions

MALDI-IM- σ TOFMS is an excellent instrumental platform for measurements of NCX since it produces predominantly singly charged ions; moreover, the 2 Torr operating pressure of the IM cell allows rapid manipulation of proteins on a time scale which allows imaging and other high throughput applications from samples in which no other separations is either possible or desirable (e.g., tissue imaging, rapid clinical analysis). Moreover, the ability to probe portions of the neutral plume with different lasers allows post-ionization that can be correlated shot by shot with the evolution of each MALDI ion from specific surface locations. More sophisticated placements of the lasers before, within, and after the IM cell may ultimately enable the gas phase equivalent of an enzymatic digestion of the molecules emanating from a specific region on a sample surface. The inherent tolerance of MALDI to salt adduction combined with imaging mass spectrometry allows in depth study of the effects of ionic strength on the formation (and possible segregation) of cationized NCX.

The experimental protocol which has been outlined in this paper should yield *ex-situ* relative gas phase binding stabilities which might be predictive of a candidate molecules *in vivo* propensity to form NCX with a particular receptor site. Generally utility of this approach as a screening tool awaits further correlations between gas phase binding stability and efficacy in animal human studies. However, some hope for this approach exists, since the gas phase NCX stability of specific epitopes has already been correlated with their biological efficacies in two different animal studies [38].

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute on Drug Abuse, NIH. The authors thank Dr. Damon Barbacci for helpful comments and the Office of National Drug Control Policy (ONDCP) for instrumentation funding, without which this and other projects could not have been accomplished. This project has also been funded in part at Ionwerks with federal funds from the National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, under SBIR Phase II Contract No. HHSN271200900009C and SBIR Phase II Grant No. 1RC3DA031431-01

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Figure1.

a) Epitopes location within D₂R and A_{2A}R. b) Charge/pH plot of the A_{2A}R-D₂R epitopes c) Model of the docking of the A_{2A}R-D₂R epitopes.

Figure 2. Schematic of MALDI-IM-oTOFMS showing three lasers within the MALDI source region. One YLF (349nm) is used for MALDI and simultaneous neutral desorption. The second laser depicted is an excimer laser (F2 at 157 nm) which creates ions from the otherwise lost neutrals by direct photoionization and photofragmentations. A third laser (*e.g.* YLF 349 nm) could have been used for a second postionization of neutrals by photodesorbing gas phase neutral analyte matrix complexes to create analyte ions (gas phase MALDI).

Figure 3.

MALDI-IM-oTOFMS 2D color intensity plot of the m/z peaks and drift times from a mixture of VLRRRRKRVN (ppt 1) +SAQEpSQGNT (ppt2). a) spectrum showing the MH^+ and of the various peptides and NCX, and their distribution along the peptide trend line. Note that the basic and acidic peptides as well as the noncovalent complex travel along separate trend line indicating a change in conformation that takes place when the NCX is formed. b) Shows in greater detail, the *in-source* addition of 2 H_3PO_4 to ppt 1 and the

subsequent loss of on phosphate upon entering the low pressure region between the IM cell and the oTOFMS.

Figure 4.

NRRRVEAAARR (ppt 3)+pSEDDDDQpSVSED (ppt 4) yield multimer NCXs. a) Spectrum showing the MH⁺ and MH²⁺ of the various peptides and NCX, and their distribution along the peptide trend line. b) Shows the addition of H₃PO₄ to ppt 3, and how its peak is easily separated from ppt 4 as they travel along different trend line.

Figure 5. Calmodulin and VLRRRRKRVN (ppt 1) from stable NCX. a) shows the MH⁺ (and multiple charge states) of CaM and the NCX and their multimers b) is an expansion around the CaM and NCX showing the effect of DHA matrix adduction.

Figure 6.

Postionization of neutral peptide/CaM NCX. a) Four dimensional data showing MALDI and Neutral postionized trend lines. b) Line shape comparisons between MALDI and Postionized CaM and CaMppt5_{1,2} NCX complexes. The total ion counts for MALDI and postionization are almost identical, but significant fragmentation broadens the VUV postionization spectrum.

Figure 7.

a) IMMS spatial imaging of total sum of all signal associated with **ppt 4** (**A**) and **ppt 3** (**B**) compared to all signals (protonated and adduct) associated with the **NCX**. Image (A+B) are from an m/z window over the range 1200 to 1500 amu and Image (NCX) over the NCX region at 2600 to 2700 amu.

Figure 8.
A derived image montage from singly charged protonated or sodiated peptides correlated for comparison with protonated or sodiated NCX.