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ETD and Sequential ETD localize the Residues involved in D2-A2A Heteromerization

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Abstract

Certain amino acid residues and posttranslational modifications play an important role in the formation of noncovalent complexes (NCXs) by electrostatic interactions. Electrospray ionization mass spectrometry (ESI-MS) is the most widely used MS technique for the study of NCXs, due to its softer ionization process and compatibility with the solution phase of NCX mixtures. In order to locate the site where interactions are forming in NCXs involving phosphopeptides and adjacent arginines, tandem mass spectrometry studies using collision-induced dissociation (CID) and electron transfer dissociation (ETD) were performed on NCXs at different charge states. CID fragmentation revealed two dissociation pathways: one in which the electrostatic interaction is disrupted and another in which the covalent bond attaching the phosphate group to the amino acid residue is cleaved, while the electrostatic interaction is maintained. ETD and sequential ETD/ETD, and CID/ETD allow the determination of the NCX interaction site. These results confirmed the involvement of the phosphorylated amino acid and at least two adjacent arginines as the binding site.

Introduction

Noncovalent complexes (NCXs) are formed from a wide variety of interactions that includes hydrophobic interactions[1], electrostatic (coulombic) interactions[2,3], and hydrogen bonding[4,5], which is actually a weak electrostatic interaction[6]. Electrostatic interactions are of particular importance in determining the quaternary structure of interacting molecules and determining their conformation which gives specificity to the proteins involved[7] such as in receptor heteromerization[8,9]. Certain amino acid residues and posttranslational modifications play an important role in the formation of NCXs by electrostatic interactions[6,10,11]. The use of mass spectrometry to study NCXs in biological systems has sky rocketed in recent years[6,12,13]. Mass spectrometry is well-suited to study electrostatic interactions as they are strengthened and stabilized in the gas phase when compared to solution phase, due to the dielectric constant of the solvent, which weakens electrostatic interactions in solution phase[6,14]. Electrospray ionization mass spectrometry (ESI-MS) is the most widely used MS technique for the study of NCXs, due to its softer ionization process and compatibility with the solution phase of NCX mixtures. ESI-MS has been successfully used to analyze protein complexes[14-16], protein-peptide complexes[17,18], NCXs of peptides[19-21], NCXs of DNA and RNA[22-24], and NCXs in drug discovery[25].

Several studies using mass spectrometry have probed the electrostatic interaction forming NCXs to determine its location and stability/strength. First, NCXs enzymatic digests of NCXs have been conducted to determine the possible binding sites for peptide-peptide[2,26] and protein-peptide[27] interactions. However, the interpretation of results can be complicated due to the plethora of peaks and background ions resulting from the digest, although it narrows down the possible location of the electrostatic interaction. Tandem MS experiments using collision-induced dissociation (CID) have analyzed the gas-phase stability of NCXs of different molecules, in order to compare the strength of the noncovalent bond holding complexes together[20,21,28,29]. Furthermore, results from studies analyzing the fragmentation of NCXs formed with phospho/sulfopeptides, using CID revealed two dissociation pathways for these complexes: one in which the electrostatic interaction is disrupted leading to the two intact partners and another in which the covalent

bond attaching the phosphate/sulfate group to the amino acid residue is cleaved, while the electrostatic interaction is maintained[19,21,30]. Similar dissociation pathways in which the noncovalent bond was maintained, has been observed in NCXs between oligonucleotide/polybasic compounds[31], ribonuclease-nucleotide[32] and DNA/peptides[33]. While these results confirm the role of certain amino acid residues and chemical groups (including phosphate/sulfate), in the formation, stability and strength of NCXs, they do not allow the assignment of the specific sites involved in the electrostatic interactions, especially when more than one possible binding site is present on the domains involved.

CID fragmentation can be complemented by using either electron capture dissociation (ECD) or electron transfer dissociation (ETD), which cleave the peptide backbone while side chains and post-translational modifications, such as phosphorylation, are left intact[34-36]. In one previous study, ECD was conducted on weakly bound polypeptide complexes and produced some intramolecular fragmentation ions without weak bond dissociation[37]. Our group showed how ETD can be an effective tool for the identification of the specific amino acid residues involved in electrostatic interactions (NCXs) between peptides[38]. ECD has recently been used to elucidate the site of binding between ATP and protein[39]. In this study, we investigated the interaction between epitopes from D2 and A2A receptors, known to be biologically involved via receptor heteromerization[9,40]. Both CID and ETD were conducted on the NCX, and the use of sequential ETD experiments on NCX fragments will be introduced to identify the site of interaction when multiple sites of interaction are possible.

Materials and Methods

Chemicals

The basic epitope **RRRRKR**VNT**KR**SS**R** (**R_4**), [$C_{73}H_{142}N_{38}O_{19,}$ 1855.13 u] a linear motif in the third intracellular loop of the D_2 receptor, and the acidic epitope SAQE**pS**QGNT (**pS**), [$C_{34}H_{59}N_{12}O_{19}P_{,}$ 1000.35 u] a linear motif located in the carboxy terminus of the Adenosine A_{2A} receptor, where p denotes a phosphorylated residue, were synthesized at the Johns Hopkins School of Medicine Sequencing and

Synthesis Laboratory (Baltimore MD). EtOH was purchased from the Warner-Graham Company (Cockeysville, MD).

Sample Preparation and Analysis

Peptides stock solutions were prepared in water at a concentration of 1 nmol/μL. A mixture of 1:4 (basic:acidic) ratio was prepared and diluted in 50% ethanol prior to mass analysis.

Mass Spectrometer

Analysis was conducted on a LTQ Orbitrap Velos mass spectrometer (Thermo Electron, San Jose, CA) with a static nanospray source in positive ion mode. A spray voltage of 1.5 kV was used with 2 μm nanospray tips (New Objective, Woburn, MA). A mass selection window of 2 mass units was used for MS^n analysis. CID experiments were conducted with collision energies of 12 to 17. ETD experiments were conducted via a modification involving a chemical ionization source at the rear of the instrument. Fluoranthene ions were used as the ETD reagent. The anion target was $5e^6$ and the activation time for the ETD reaction was 100 ms. All mass spectra in this study, are the sum of 25 to 50 scans. The mass resolution was set at 60,000 for an *m/z* 400. The MS^3 sequential experiments were performed in the linear ion trap. To simplify notations, protonated species $[M+nH]^{n+}$ are labeled as M^{n+}.

Results and Discussion

In order to investigate the stability of the interaction involved in NCX formation, a mixture of two peptide R_4 and pS, known to form complexes, was analyzed by ESI-MS. Figure 1A shows an ESI-MS spectrum for a sample mixture of R_4 and pS in positive ion mode. The major peaks observed correspond to the peptide R_4 with 3 to 6 charges and pS with one charge. The multiple charges on R_4 are expected since it contains seven arginines residues. The NCX formed between R_4 and pS is also observed with 4 to 6 positive charges. Figure 1B shows the CID MS/MS spectrum for the NCX^{4+}observed in Figure 1A at *m/z* 715.13 Da. The dissociation of the NCX^{4+} peak resulted in a singly charged ion corresponding to pS (*m/z* 1001.36 Da) and a very intense triply charged ion attributed to R_4^{3+} (*m/z* 619.38 Da). This result suggests an uneven sharing of charges between the two partners of the complex when disrupted by CID. The positive charges stay mainly on the basic epitopes. These fragment ions result from the disruption of the electrostatic interaction between

the two peptides. A second pathway, cleaving the covalent bond between the oxygen and the phosphate lead to the formation of the ion fragments at *m/z* 646.04 Da corresponding to $[R_4+HPO_3]^{3+}$, and $[pS\text{-}HPO_3]^+$ at 921.39 Da. This type of fragmentation for NCXs has been reported in previous studies[19,21,30,41]. An additional fragment ion from the acidic $[pS\text{-}H_3PO_4]^+$ at 903.38 Da can be observed as a consecutive loss of water from $[pS\text{-}HPO_3]^+$ or the loss of H_3PO_4 from pS^+.

The ETD product-ion spectrum from the NCX^{4+}, in Figure 1C, shows a very different fragmentation of the complex compared to the CID. Unlike CID, the NCX did not dissociate into the parent peptides, but mainly produced c and z ions, fragmenting along both peptides. Intramolecular fragments which conserved the electrostatic bond have been marked with an asterisk *. These fragment ions allowed the identification of the amino acids involved in the binding. For the basic peptide (R_4) the ions of the c serie (c^*_4- c^*_{13}, color purple) and the z serie (z'^*_{12} - z''^*_{14}, color pink), that were interacting with the phosphate, all contained the two adjacent arginines (Arg_3 (3^{rd} arginine of the peptides) and Arg_4). Likewise, the fragment ions from the acidic peptide (pS): c^*_5 - c^*_8 (color orange) and z'^*_5 - z'^*_9 (color green) that were interacting with R_4, contained the phosphoserine. Other complementary fragment ions from R_4 are also present in the spectrum (c and z' in blue) but do not offer any information on the interaction site of the complex.

CID and ETD experiments were also conducted on both NCX^{5+} and NCX^{6+}(Figure 2). The CID spectra again show two fragmentation pathways. The first one is the complex dissociation, leading to pS at *m/z* 1001.36 and R_4 at m/z 464.79 in Figure 2A (corresponding to R_4^{4+}) or at *m/z* 372.03 in Figure 2B (corresponding to R_4^{5+}). As observed with NCX^{4+}, the charges are not evenly shared in this complex as most of them are on the basic epitope. For every charge state tested, pS is singly charged and R_4 is multiply charged (one charge less than the complex), which is understandable considering the number of arginines in R_4. Finally, as for the NCX^{4+}, another fragmentation pathway is observed, in which an additional mass of 79.97 u (corresponding to HPO_3) is seen on the basic peptide $[R_4+HPO_3]$. The relative intensity of this peak (R_4+HPO_3), compared to R_4, shows a net decrease when the number of positive charges increase. An

explanation, that we proposed[19], is that as the number of charges on the complex increases the stabilization of the guanidinium group and decreases the deprotonation of the phosphate group, thus weakening the electrostatic bond.

ETD spectra were also recorded for NCX^{5+} (Figure 2C) and NCX^{6+}(Figure 2D). Similar to the ETD mass spectrum of the NCX^{4+} ion (Figure 1C), numerous ion fragments are observed from the cleavage of peptide bonds in both epitopes (Supplementary data). Ion fragments from the basic peptide which is still interacting with pS (c^{*}_{4} - c^{*}_{13} and z'^{*}_{12} - z'^{*}_{14} respectively in purple and pink) and their complementary fragment ions (c_1 - c_2 and z'_2 - z'_{10}) are observed. Basic peptides fragments still involved in the interaction always contain Arg_3 (3rd arginine of the peptides) and contain at least two adjacent arginines. To form the complex, we previously showed that two arginines were required[10], these results confirm it and show that they are essential for the complex stability. Overall ETD fragments from R_4 are more intense with the $NCX^{6+}$and NCX^{5+} than with the NCX^{4+}, this can be explain by the fact, that as observed in CID, and as expected, most of the charges are on the basic epitopes. These charges allow us to better see the fragment ions from R_4 by mass spectrometry and make the cleavage by ETD easier. However, when looking at the acidic peptides, ETD results in fewer fragments (Figure 2C and 2D). Ion fragments (c^{*}_{8} and z'^{*}_{6} - z'^{*}_{9}) still interacting with R_4 are seen in both spectra. The diagnostic ion c^{*}_{7} is present only in the ETD spectrum of NCX^{6+}. Surprisingly, we have less coverage on the acidic peptide than with the NCX^{4+}. This lack of fragmentation from the acidic peptide is an issue in determining the binding site and is explained by the number of charges increased on the basic epitope.

To have access to the binding site, MS^3 sequential ETD (ETD/ETD) were attempted on the ETD-fragment $[c_4+SAQEpSQGNT+2H]^{2+}$ at m/z 821.90 from the $[NCX+5H]^{5+}$ (Figure 3A). The parent ion is only doubly charged, for a better visibility of the intensity of the fragments the mass range 1000-1700 has been multiplied by 3. C- and z-ions fragments from the acidic peptide (c^{*}_{5} - c^{*}_{8} and z'^{*}_{5} - z'^{*}_{8}) where the interaction was intact with c_4 from R_4 are seen. This pattern correspond exactly to the one obtained with the

$[NCX+4H]^{4+}$(Figure 1C), confirming that the phosphoserine is the preferred interaction site with the c_4 fragment. C_4 is able to lose one of the arginine from each side of the chain. The same experiment was conducted on the ETD-fragment $[c_3+SAQEpSQGNT+2H]^{2+}$ at m/z 743.85 from the $[NCX+6H]^{6+}$ confirming the phosphoserine as the preferred site of interaction (data not shown). Sequential ETD of the ion fragment $(R_4+HPO_3)^{4+}$ from the CID of NCX^{5+} was also performed to confirm the binding site on the basic epitope. The CID/ETD spectrum is presented in Figure 3B, and shows the cleavage of peptide bonds on R_4. Ion fragments still interacting with the phosphate (c^*_3 - c^*_{13} and z'^*_{12} - z'^*_{14} respectively in purple and pink) and their complementary fragment ions (c_1 - c_3 and z'_2 - z'_{10}) are observed. The same series of ions are observed than in Figure 2A and 2B, confirming the Arg_3 as the most favorable site on the basic epitope. All ETD fragment ions, conserving the interaction, possess at least two adjacent arginines.

Conclusions

CID experiments show that the electrostatic interaction is so stable that often a covalent bond will break before the electrostatic bond is disturbed .This work demonstrates the utility of ETD for studying NCX by generating intermolecular fragments from both partners. Similar to CID, ETD spectra are highly dependent on the charge states of the parent ions. The comparison of the product-ion mass spectra at different charge states shows less information at higher charges as it leads to less fragmentation from the acidic partner. To overcome this issue, the determination of the interaction site of the NCX has been done using sequential ETD/ETD. We confirmed the involvement of the phosphorylated amino acid at every charge state. ETD and sequential CID/ETD confirmed the arginines as the binding site and the necessity of at least two adjacent arginines. Future work will include using ETD and sequential ETD to probe for the interaction sites in more complex NCXs involving proteins.

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Figures

Figure 1. Ion mass spectrum (A) from a peptide mixture of SAQEpSQGNT(pS) and RRRRKRVNTKRSSR(R_4) and product-ion spectra of the $[NCX+4H]^{4+}$with (B) CID and (C) ETD.

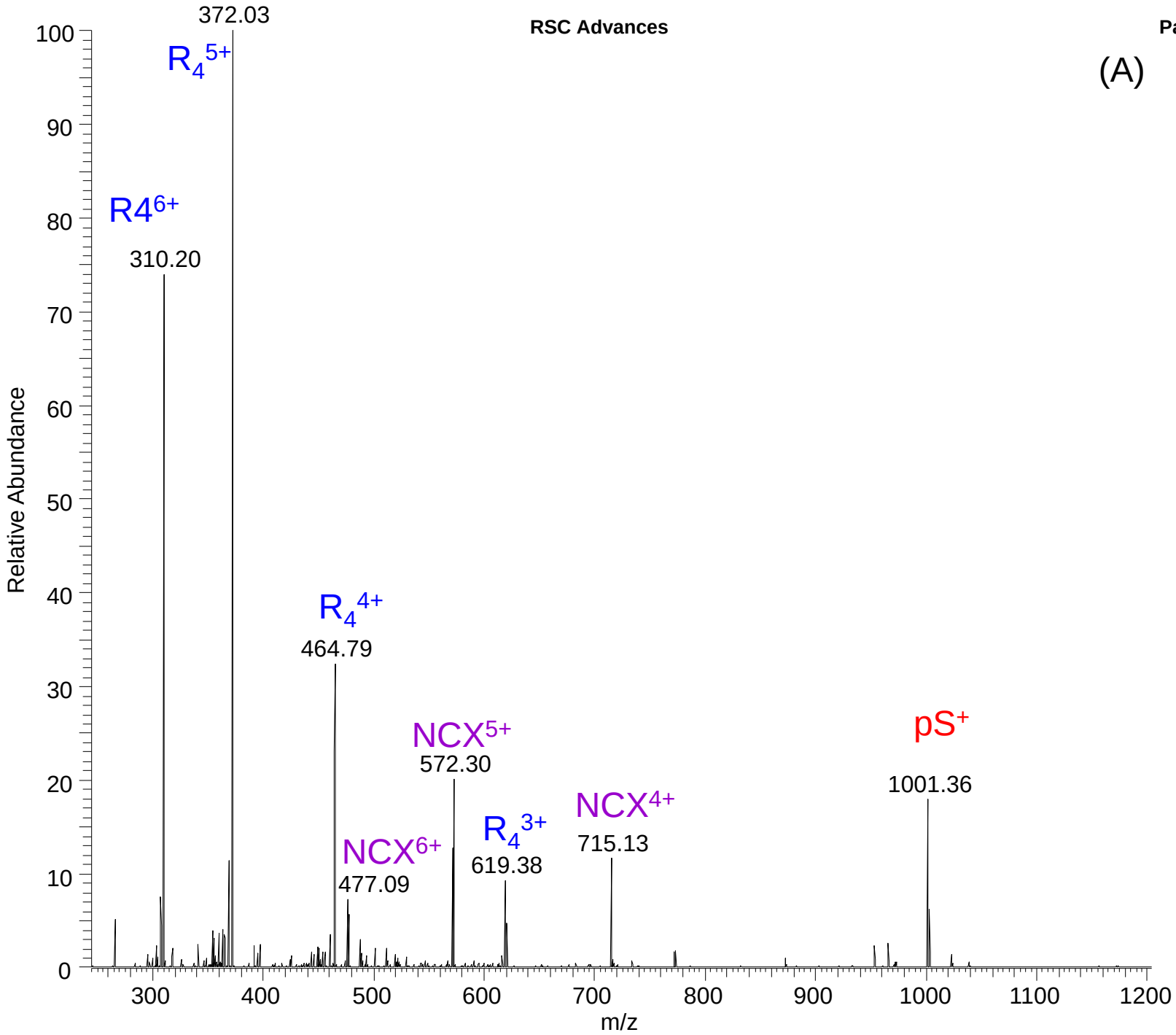
Figure 2. CID-product-ion spectra from a peptide mixture of SAQEpSQGNT and RRRRKRVNTKRSSR of the (A) $[NCX+5H]^{5+}$ and the (B) $[NCX+6H]^{6+}$. ETD-product-ion spectra of (C) $[NCX+5H]^{5+}$and $^{+}$(D) $[NCX+6H]^{6+}$.

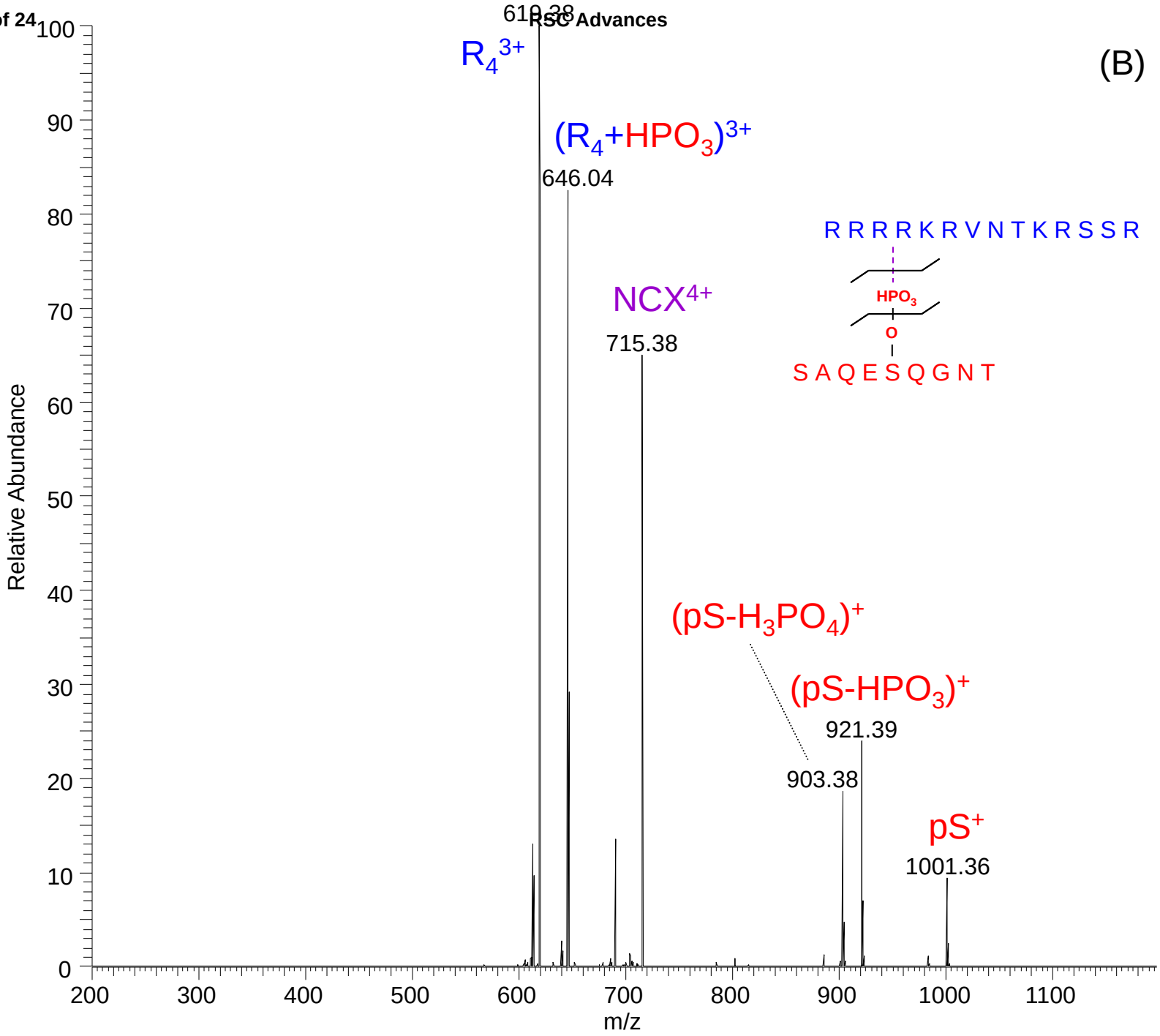
Figure 3. (A) Sequential ETD/ETD-product-ion spectra from $[c_4+SAQEpSQGNT+2H]^{2+}$ at m/z 821.90 from the ETD of the $[NCX+5H]^{5+}$. (B) Sequential CID/ETD-product-ion spectra of the $[RRRRKRVNTKRSSR+HPO_3+4H\ ]^{4+}$ at m/z 484.78 from the CID of the $[NCX+5H]^{5+}$.

Figure ETD

Figure 1

- Ion mass spectrum (A) from a peptide mixture of SAQEpSQGNT(pS) and RRRRKRVNTKRSSR(R_4) and product-ion spectra of the $[NCX+4H]^{4+}$with (B) CID and (C) ETD.





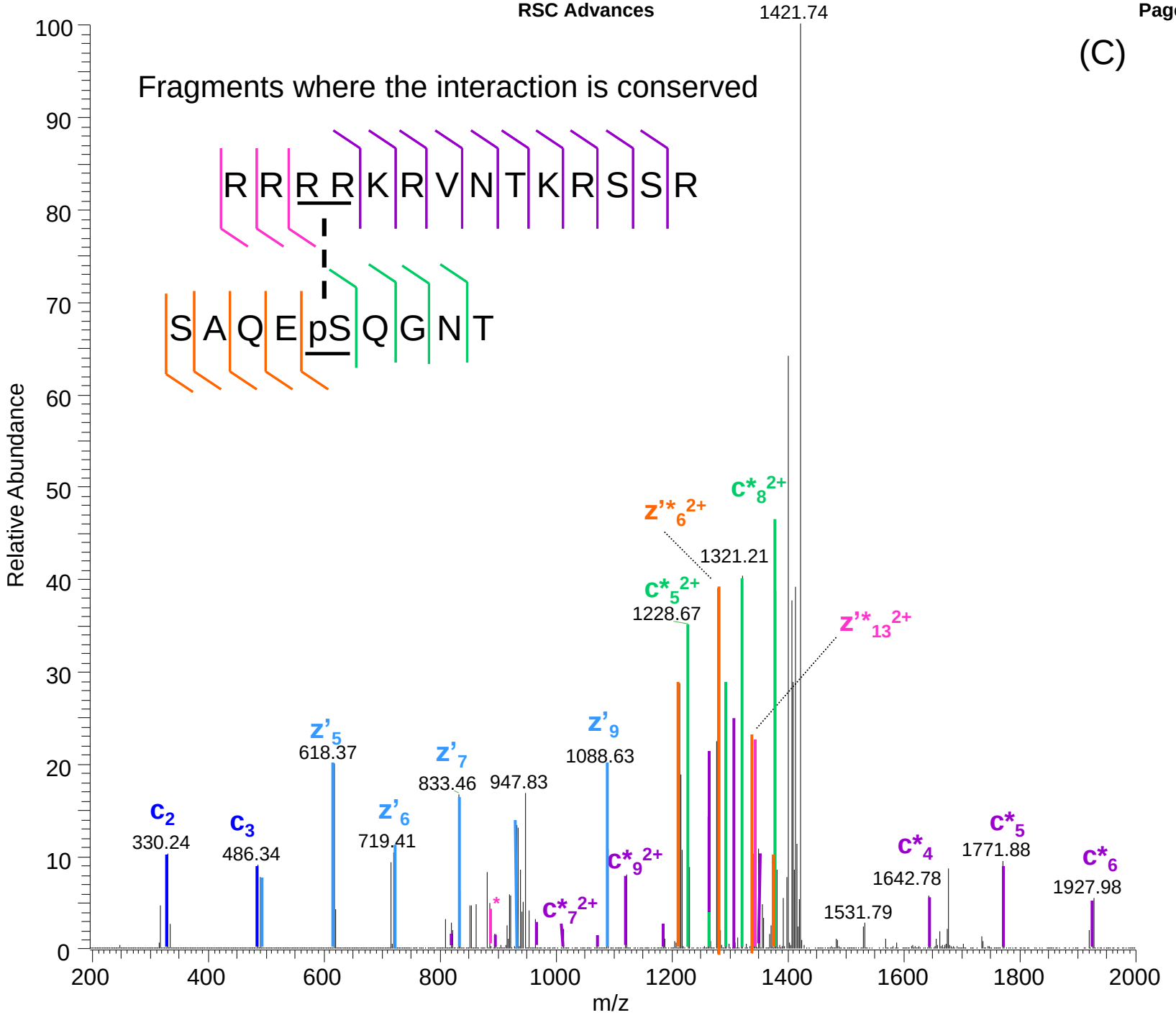
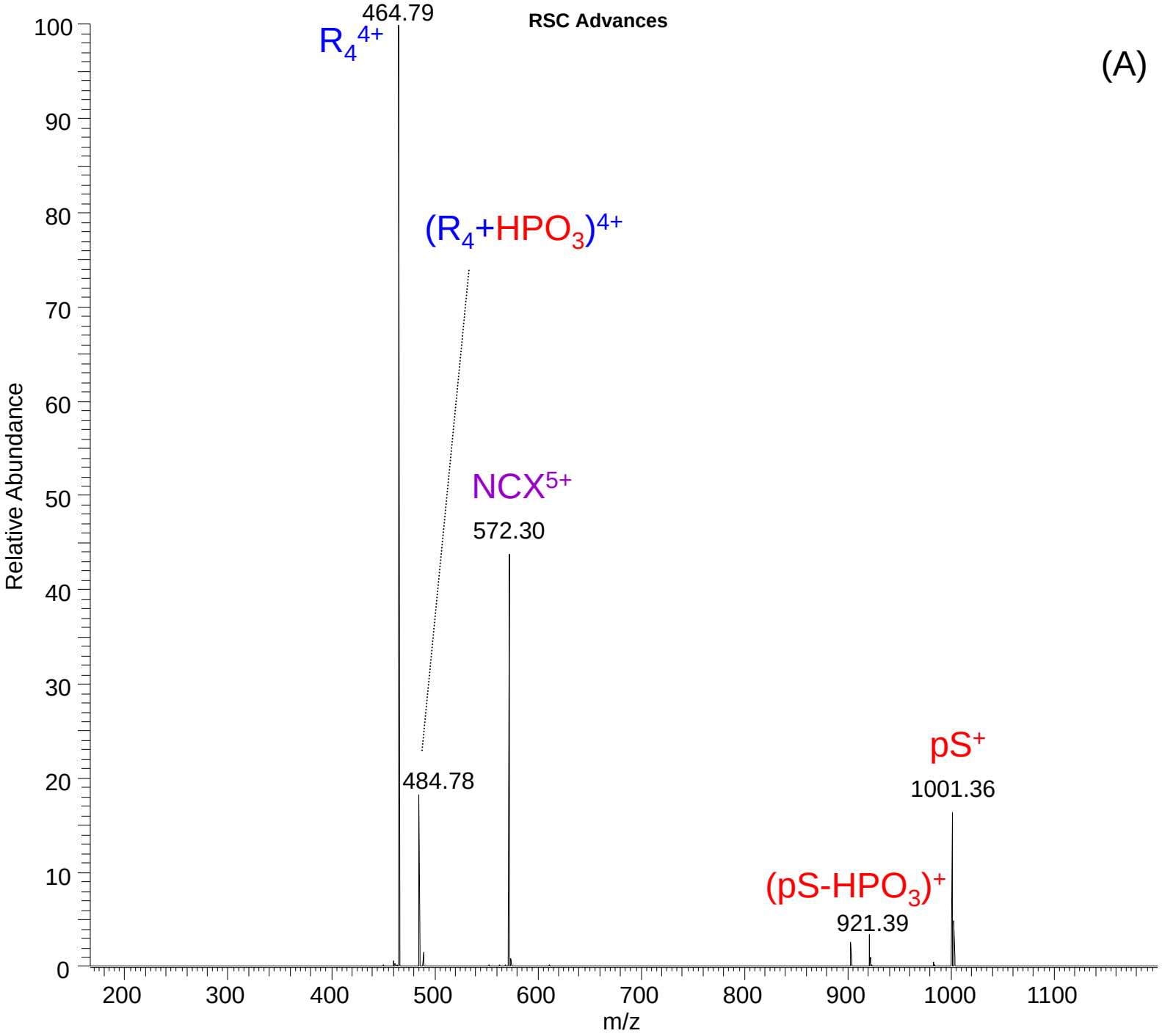
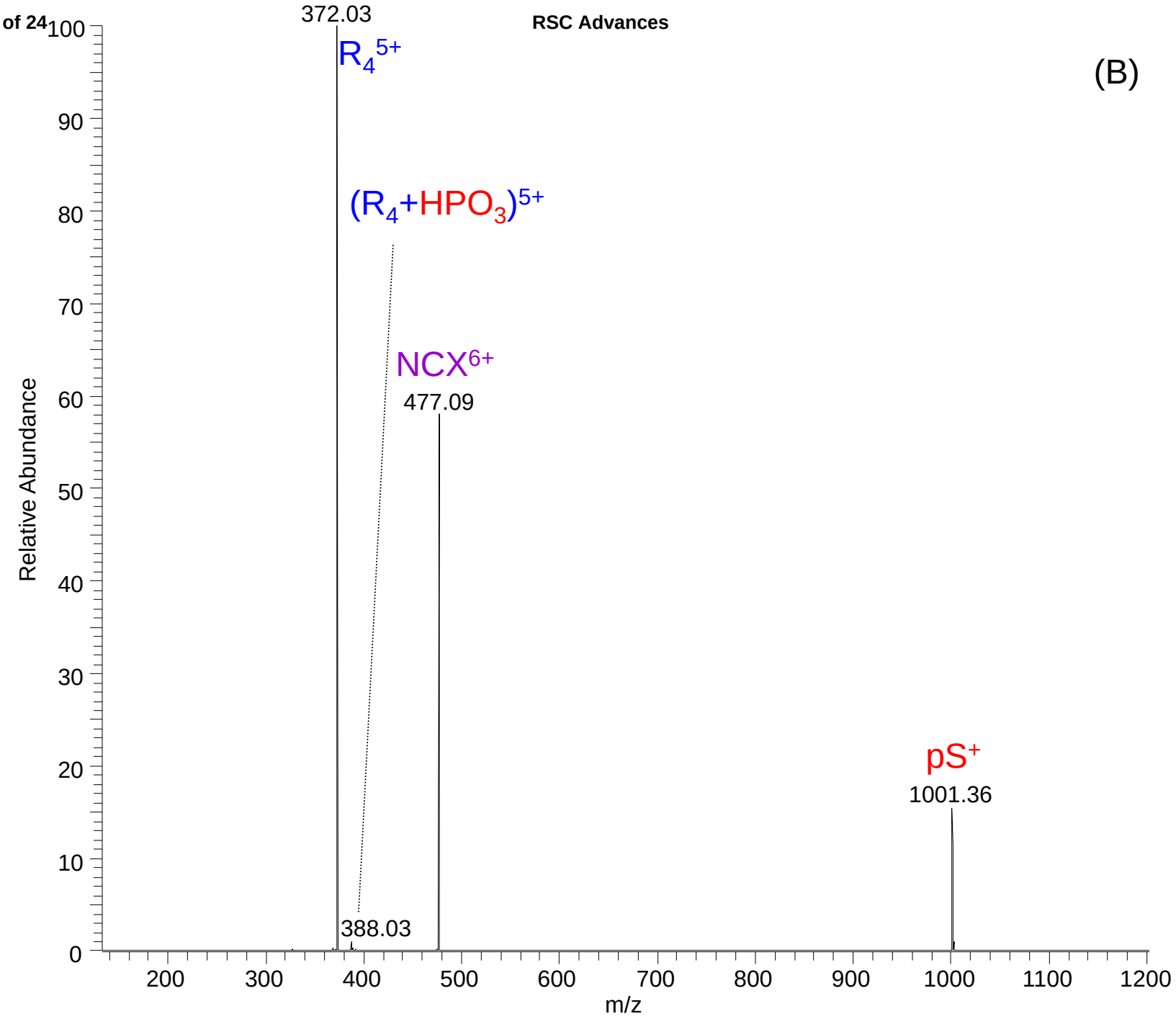
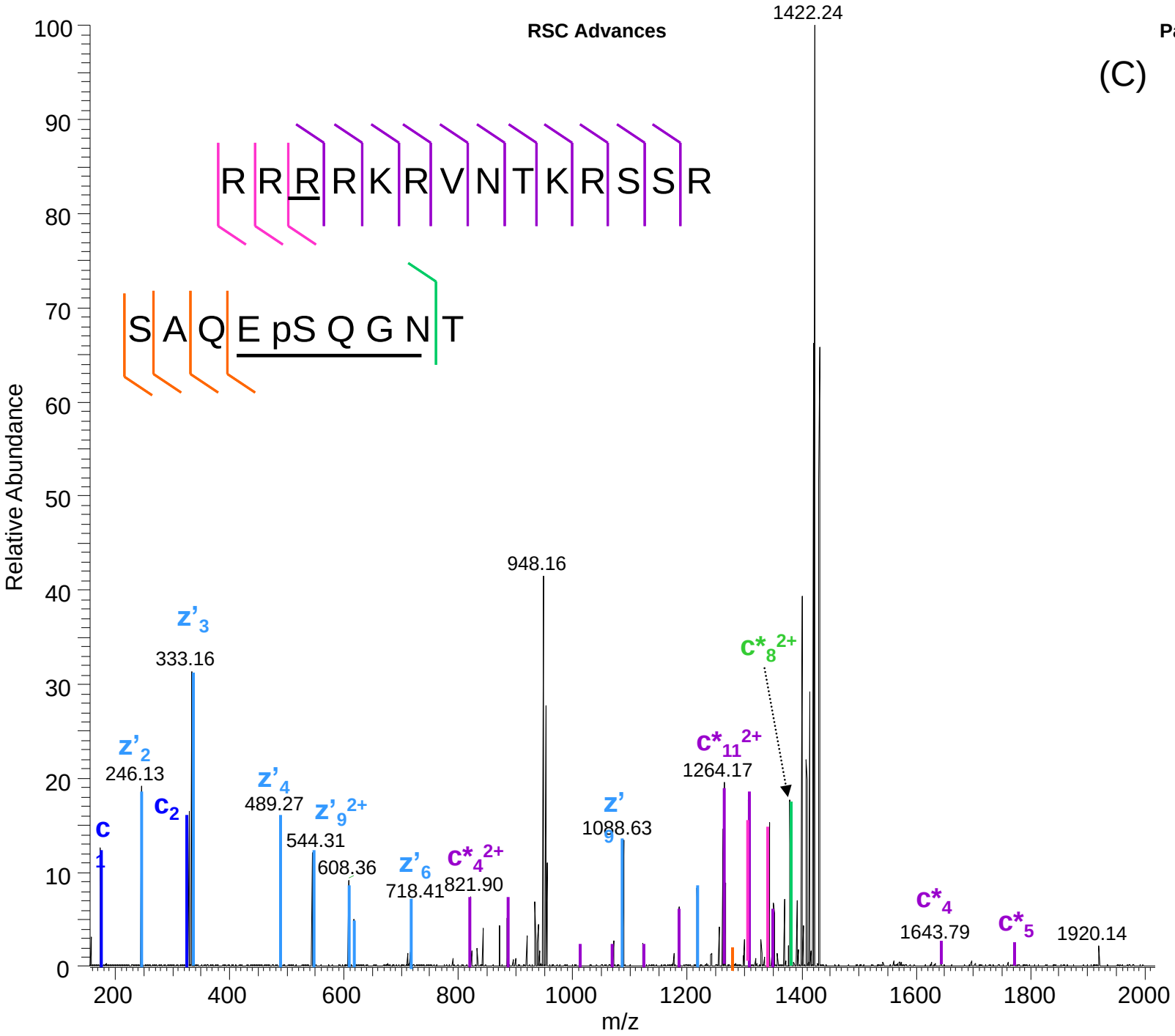


Figure 2

- CID-product-ion spectra from a peptide mixture of SAQEpSQGNT and RRRRKRVNTKRSSR of the (A) $[NCX+5H]^{5+}$ and the (B) $[NCX+6H]^{6+}$. ETD-product-ion spectra of (C) $[NCX+5H]^{5+}$and $^{+}$(D) $[NCX+6H]^{6+}$







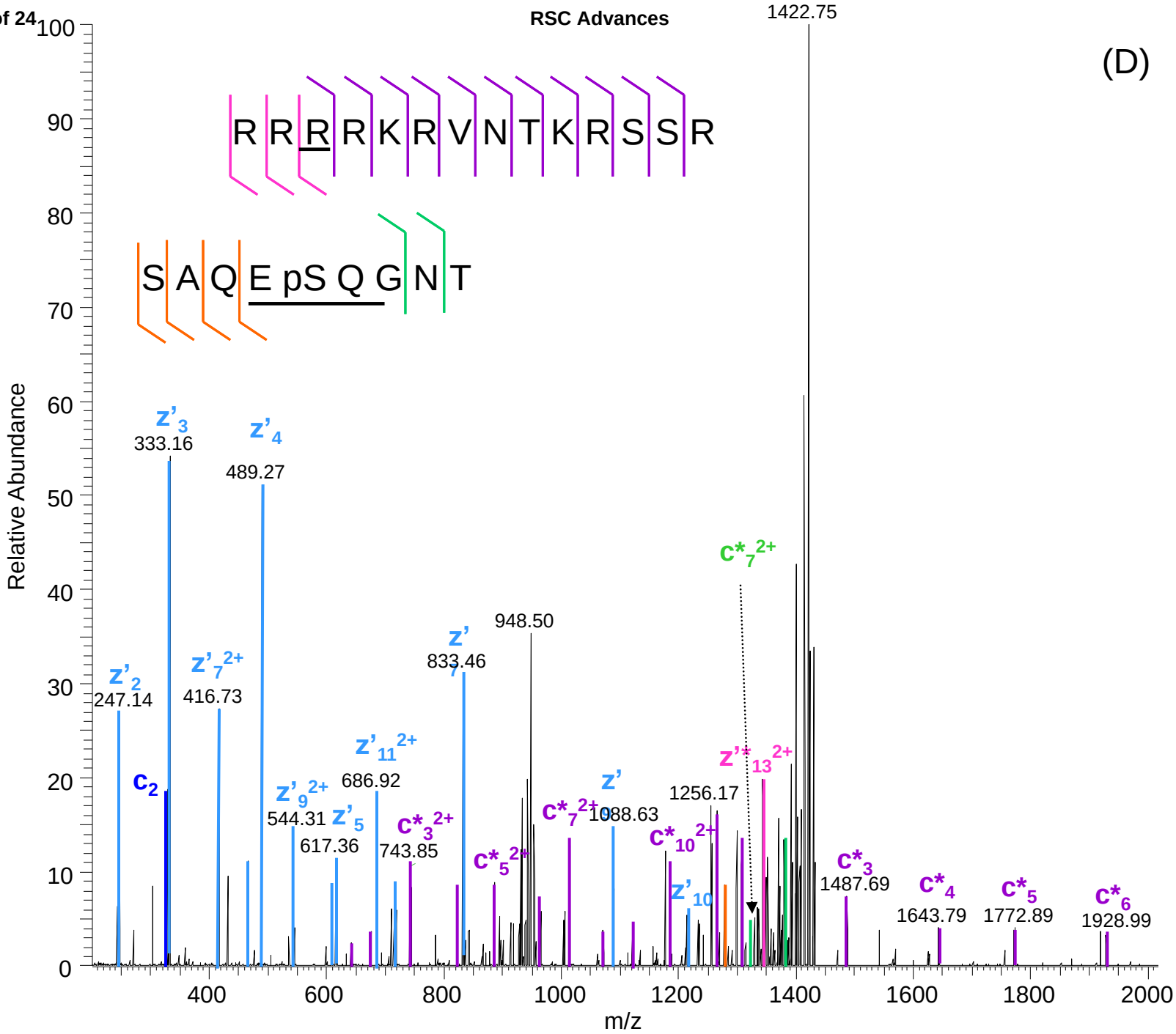


Figure 3

- (A) Sequential ETD/ETD-product-ion spectra from $[c_4$+SAQEpSQGNT+2H$]^{2+}$ at m/z 821.90 from the ETD of the $[NCX+5H]^{5+}$. (B) Sequential CID/ETD-product-ion spectra of the $[RRRRKRVNTKRSSR+HPO_3+4H\ ]^{4+}$ at m/z 484.78 from the CID of the $[NCX+5H]^{5+}$