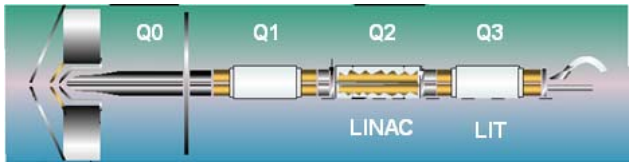
True CID Fragmentation in a Collision Cell Produces Superior MS/MS for Automated Characterization of Phosphorylation Sites

The dynamic phosphorylation of proteins often plays a key role in regulation of protein activity or signaling pathways where one or many phosphorylation sites are involved. The detection and characterization of the specific phosphorylation sites is important in understanding these biological processes. Strategies, such as precursor ion scanning[1] or IMAC enrichment[2] followed by LCMS, are used to specifically detect the phosphorylated peptides. Once detected, it is important to then generate high quality MS/MS spectra on each phosphopeptide to determine the exact site of modification. Knowing the exact site of phosphorylation enables quantitative monitoring of changes in the modification at that site as a result of the biological processes. Using mass spectrometers such as the Q TRAP® or QSTAR® LCMSMS systems, where MS/MS is performed in a LINAC® collision cell, the fragmentation spectra produced is rich in this essential information.

Mechanism of Fragmentation of Phosphopeptides in a Collision Cell

Phosphopeptides are selected for MS/MS in the first quadrupole Q1 of a Q TRAP® or QSTAR® system. RF/DC isolation in the quadrupole reduces the risk of fragmentation of fragile precursor ions. As the phosphopeptide is then accelerated into the higher pressure LINAC® collision cell, the ions collide with nitrogen gas molecules with energies between 20-100 eV. The ion paths of the 4000 Q TRAP® system and QSTAR® system are shown in Figure 1 and illustrate this configuration. A single higher energy collision leads to loss of the phosphate group from the peptide or fragmentation across the peptide backbone. Then, secondary fragmentation events can occur, such as subsequent loss of phosphoric acid from the backbone fragments.

4000 Q TRAP® System Ion Path



QSTAR® System Ion Path

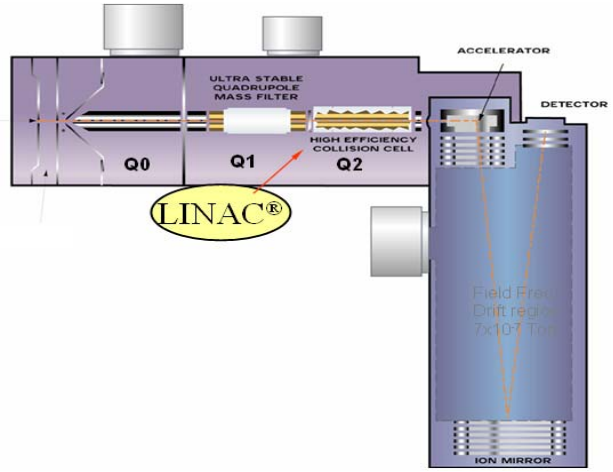


Figure 1. Ion paths of the Q TRAP® system and QSTAR® system. Because of the hybrid nature of these mass spectrometers, MS/MS is performed in a unique way. Mass isolation is done by using RF/DC on the Q1 quadrupole to pass a single peptide ion into the Q2 LINAC® collision cell. Here the peptides collide with gas molecules and fragment. The fragment ions pass through into either the Q3 linear ion trap mass analyzer, or into the time-of-flight mass analyzer.

Characteristically, the spectra have a y-ion series that extends across much of the sequence. In addition, neutral loss fragments can often be seen from the y-ions that possess serine or threonine phosphorylation, however these do not dominate the spectra. These ions help localize the actual site of phosphorylation as shown in Figure 2. The resulting phosphopeptide MS/MS spectra are much easier to interpret and allow localization of the phosphorylation site, especially with automated protein identification software.

The Most Information Rich MS/MS Spectra Are Obtained with Collision Cell Fragmentation

The examples shown here in Figures 2 and 3 are from experiments on the 4000 Q TRAP® system, however similar fragmentation patterns would be observed on the QSTAR® system. The higher energy fragmentation patterns due to MS/MS in the collision cell lead to richer product ion spectra. More informative MS/MS data allows for the exact location of the phosphorylation site to be defined more often.

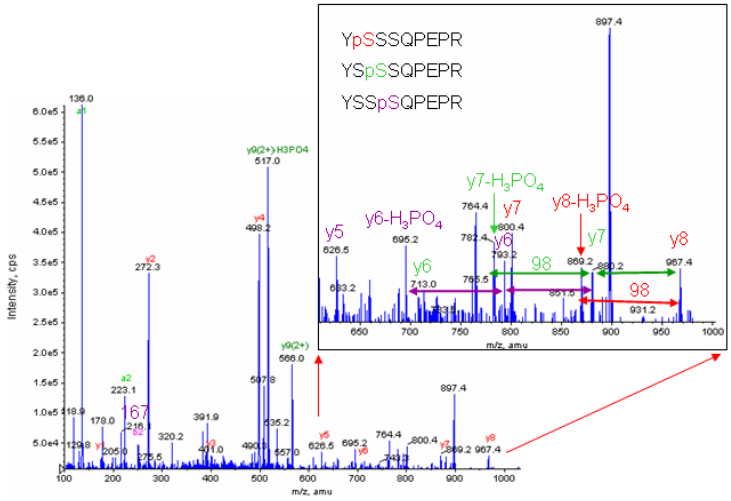


Figure 2. MS/MS spectrum from a 4000 Q TRAP system for the kinase CHK1. The fragment ion spectrum for this phosphorylated peptide contains evidence for 3 isobaric co-eluting phosphopeptides. In this case, the peptide sequence has three serines in a row, each can be singly phosphorylated. Because of the high quality MS/MS, there is strong evidence in support of all three phosphorylated forms in this one spectrum.

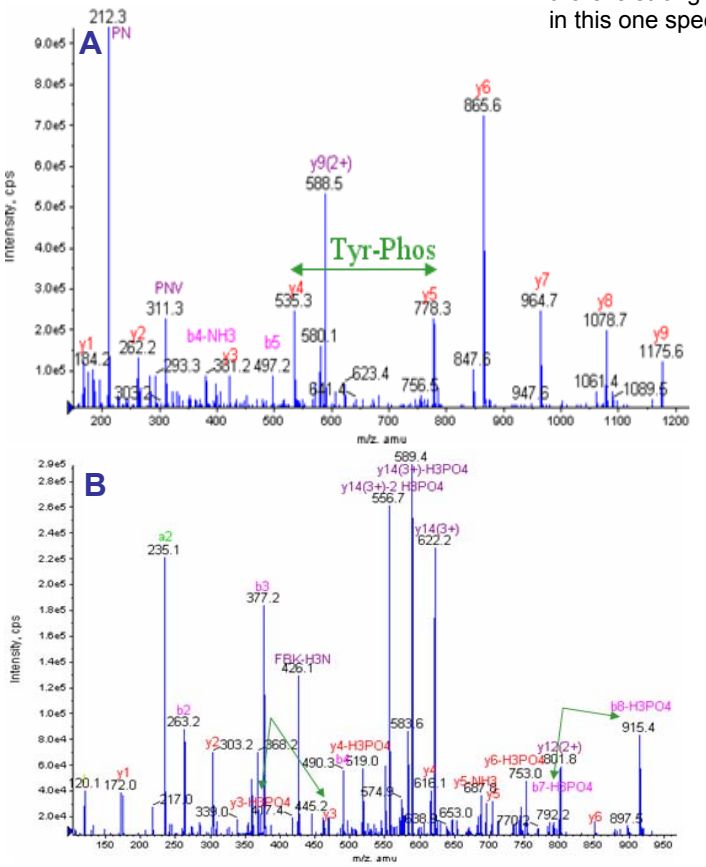


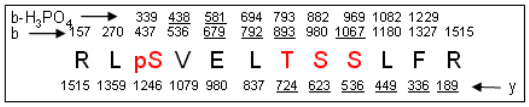
Figure 3. MS/MS spectra from a 4000 Q TRAP system. (A) Peptide GEPNVS**pY**IDSR for the kinase GSK3β. The fragment ion spectrum for this tyrosine phosphorylated peptide clearly shows the location of the phosphorylated residue. No neutral loss fragment ions are seen for tyrosine phosphoryation sites. (B) Doubly phosphorylated peptide DFNIKL**pS**DFSF**pS**KR from kinase TSSK1. The y-ion evidence shows location of phosphorylation site near the C-terminus of the peptide. The b-ion evidence indicates the second phosphorylation is on the serine towards the N-terminus.

In the example shown in Figure 2, three separate phosphopeptide sites can be determined from a single MS/MS spectrum. This peptide is singly phosphorylated on each of the serine residues in the peptide. Because the 3 peptides will be isobaric and co-eluting, they appear in the same MS/MS spectrum. The presence of both strong y ions and y-H_3PO_4 ions allows for the confident assignment of the three phosphorylation sites.

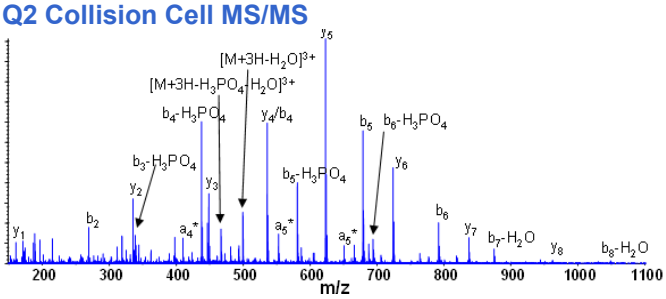
In the example shown in Figure 3, a tyrosine phosphorylated peptide and a doubly phosphorylated serine-containing peptide are shown as examples of the data quality obtained for assignment of the phosphorylation sites.

Why Resonant Excitation for Fragmentation on Ion Traps Provides Less Information

In a conventional 3D or linear ion trap, precursor isolation occurs within the ion trap. Fragile precursor ions are susceptible to fragmentation during this isolation step, reducing the MS/MS efficiency and sensitivity. During MS/MS, conventional ion traps produce fragment ions through a low energy resonance excitation process in the presence of high-pressure neutral gas. During this process, the incremental energy deposition into the peptide causes a fragmentation event where the lowest energy processes occur first. Often, the fragmentation pattern observed is dominated by ions from these lowest energy fragmentation pathways. For fragmentation of phosphopeptides, this low energy process is most often the removal of the phosphate groups or a loss of water from the peptide ion. As a result, the neutral loss of the phosphate (and/or water) from the peptide dominates the spectrum, and the peptide backbone fragments needed for site identification are very weak (Figure 4, bottom pane). In many cases, MS/MS/MS on this neutral loss fragment $[M+nH-98]^{n+}$ is required to obtain the sufficient sequence information, significantly reducing sensitivity and duty cycle.



Q2 Collision Cell MS/MS



Resonant Excitation MS/MS

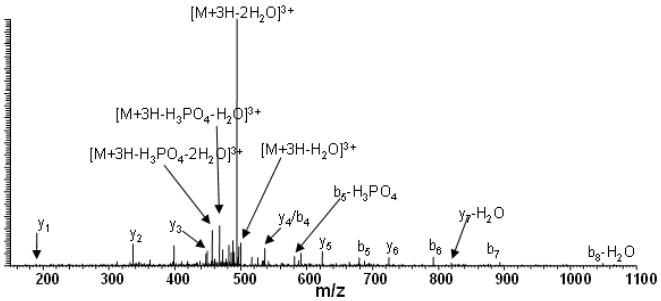


Figure 4. Comparison of the fragmentation pattern observed for a phosphopeptide (RL**pS**VELTSSLFR, methyl ester, m/z 505.8^{3+}) generated in a collision cell (QSTAR system) vs. a commercially available ion trap. Notice the greater proportion of sequence specific ions observed in the upper pane (inset) vs. the lower pane generated on a linear ion trap system with no collision cell.

Conclusions

- When studying phosphorylation, targeted strategies such as precursor ion scanning for detecting the low level phosphopeptides are very important.
- High quality information rich MS/MS spectra is key to determine the exact location of the phosphorylated residue in the peptide.
- Fragmentation in a collision cell produces high quality MS/MS for easy identification of phosphorylation sites
- Ion trap fragmentation does not produce as information rich spectra because of the tendency to prefer low energy pathways, resulting in predominantly neutral loss fragments.

References

1. Annan *et al.*, (2001) *Anal. Chem.* **73**, 393-404.
2. Stensballe *et al.*, (2001) *Proteomics* **1**, 207-222.