Metal Oxide-Based Enrichment Combined with Gas-Phase Ion-Electron Reactions for Improved Mass Spectrometric Characterization of Protein Phosphorylation

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Gas-phase ion-electron reactions, including electron capture dissociation (ECD) and electron detachment dissociation (EDD), are advantageous for characterization of protein posttranslational modifications (PTMs), because labile modifications are not lost during the fragmentation process. However, at least two positive charges and relatively abundant precursor ions are required for ECD due to charge reduction and lower fragmentation efficiency compared to conventional gas-phase fragmentation techniques. Both these criteria are difficult to fulfill for phosphopeptides due to their acidic character. The negative ion mode operation of EDD is more compatible with phosphopeptide ionization, but EDD suffers from a fragmentation efficiency even lower than that of ECD. Recently, metal oxides such as ZrO₂ and TiO₂ have been shown to provide selective enrichment of phosphopeptides from proteolytic digests. Here, we utilize this enrichment strategy to improve ECD and EDD of phosphopeptides. This approach allowed determination of the locations of phosphorylation sites in highly acidic, multiply phosphorylated peptides from complex peptide mixtures by ECD. For singly phosphorylated peptides, EDD provided complementary sequence information compared to ECD.

Keywords: Protein phosphorylation • determination of phosphorylation sites • electron capture dissociation (ECD) • electron detachment dissociation (EDD) • phosphopeptide enrichment • metal oxide • ZrO₂ • TiO₂ • zirconia • titania

Introduction

Reversible protein phosphorylation, occurring on serine, threonine, and tyrosine side chains, is one of the most common posttranslational modifications (PTMs) in eukaryotic cells. As a major regulatory mechanism of cell signaling,¹ protein phosphorylation and dephosphorylation are involved in numerous cellular processes, such as cell communication, cell development, and cell differentiation. Altered phosphorylation has been linked to several human diseases, including cancer.² To understand the mechanisms controlling these processes, we must precisely determine phosphorylation sites and the extent of phosphorylation in certain cell states. Mass spectrometry (MS), including various tandem mass spectrometric techniques (MS/MS), has become one of the most powerful and commonly used tools for proteomic analysis;³ however, analyzing protein phosphorylation remains a challenge due to its typically low stoichiometry and dynamic nature.⁴ Moreover, the acidity of phosphates renders phosphorylated species more difficult to be protonated than nonphosphorylated species, thereby resulting in signal suppression in positive ion mode MS. Peptides with multiple phosphorylations are particularly difficult to detect and are therefore often screened in negative ion mode.⁶⁻⁸ In a typical mass spectrometric analysis, proteins are digested with trypsin and phosphorylated peptides are selectively isolated by affinity chromatography to reduce sample complexity and ion suppression effects. Either electrospray ionization (ESI)⁹ or matrix-assisted laser desorption ionization (MALDI)¹⁰,¹¹ can be used to introduce samples into a mass spectrometer in which MS/MS fragmentation improves protein identification and determination of the location of modifications.

Immobilized metal ion affinity chromatography (IMAC)¹²,¹³ with Fe³⁺, Ga³⁺, or other metal ions is widely utilized to isolate phosphorylated peptides prior to mass spectrometric analysis, both on-line and off-line. However, IMAC isolation suffers from nonspecific binding of nonphosphorylated acidic peptides, and numerous factors affecting the isolation efficiency, thus resulting in poor reproducibility and cumbersome column preparation procedures. Metal oxide-based affinity chromatography, including TiO₂, ZrO₂, and Al₂O₃,¹⁴⁻²⁰ has been shown to provide high selectivity and high reproducibility for binding of phosphorylated peptides from complex mixtures, and thus, this strategy has been rapidly adapted and widely utilized for phosphopeptide enrichment from complex proteomic samples.

Collision-activated dissociation (CAD)²¹,²² is the most commonly available fragmentation mechanism for MS/MS experiments; however, collisional activation often results in dominant neutral loss of phosphoric acid (98 Da) from phosphorylated peptide precursor ions. This dominant neutral loss can be used...
as a specific marker for phosphopeptide identification; however, this characteristic of CAD is unfavorable in determining the location of modifications.\textsuperscript{23} Electron capture dissociation (ECD)\textsuperscript{24–27} achieved from gas-phase reactions between multiply protonated ions and low-energy electrons ($<$ 2 eV), offers advantages for PTM analysis. ECD proceeds via a charge-reduced radical intermediate, which fragments at peptide backbone $N$--$C_\alpha$ bonds to yield extensive c and z-type ions without loss of labile modifications, thereby allowing both peptide identification and precise determination of the locations of modifications. In spite of these clear advantages, ECD is not readily employed for protein phosphorylation analysis from complex mixtures because of its fragmentation efficiency is lower than that of CAD, rendering accumulation of sufficient precursor ion signal critical for successful analysis, a task that can be challenging for phosphorylated peptides because of the properties discussed above. In particular, signal suppression is exacerbated for multiply phosphorylated peptides, which often produce a very weak or no signal in positive ion mode ESI. Cooper and co-workers\textsuperscript{28} have successfully demonstrated ECD of a doubly phosphorylated peptide from 1 pmol of tryptic digest of bovine $\alpha$-casein, providing extensive sequence coverage and unambiguous determination of the locations of the two phosphorylation sites, by on-line LC and data-dependent ECD triggered by the neutral loss of 98 Da from phosphopeptides. However, ECD of highly acidic peptides with three or more phosphorylations was not realized with that approach.

Electron detachment dissociation (EDD)\textsuperscript{29} is a fragmentation technique operating in negative ion mode, which provides more facile detection of highly acidic phosphopeptides. EDD is achieved from gas-phase reactions between multiply charged peptide anions and medium-energy electrons ($>$ 10 eV). Charge neutralization of peptide anions by electron detachment forms radical intermediates that mainly fragment at peptide backbone $C_\alpha$--$C_\beta$ bonds, whereby generating c$^*$- and x-type ions. EDD has been demonstrated to fragment a tyrosine-phosphorylated peptide without loss of the phosphate group.\textsuperscript{30} However, EDD of serine- and threonine-phosphorylated phosphopeptides, known to be more labile than tyrosine-phosphorylated ones, has, to our knowledge, not been reported in the literature, although retention of labile sulfate groups was shown in the first EDD publication.\textsuperscript{29} One drawback of EDD is its fragmentation efficiency, which is lower than that of other fragmentation methods, including ECD.

Recently, Hunt and co-workers\textsuperscript{31} developed electron transfer dissociation (ETD), which allows ECD-like fragmentation in ion trap instruments. ETD is achieved by gas-phase ion--ion reactions between multiply protonated peptide ions and singly charged anions with low electron affinity, thereby producing a charge-reduced radical intermediate similar to that described above for ECD. A doubly phosphorylated peptide from data-dependent on-line nano-LC--ETD of an IMAC-enriched tryptic digest of human nuclear proteins showed cleavages at $N$--$C_\alpha$ bonds, thereby generating extensive c- and z-type ions with preserved phosphorylations. However, this peptide contained three arginine residues (greatly facilitating its positive mode ionization), which is not typical. Furthermore, ETD of larger peptides or proteins is hampered by the low resolution of the utilized ion trap mass spectrometer, although recent results of Coon and co-workers demonstrate that this problem can be circumvented by coupling ETD to an orbitrap mass analyzer.\textsuperscript{32} Hunt and co-workers\textsuperscript{33} have reported ETD of multiply deprotonated phosphopeptides formed by negative ion mode ESI. Here, ETD was induced via electron abstraction by Xe cations, which generated a- and x-type ions from $C_\alpha$--$C_\beta$ bond cleavages, similar to the fragmentation observed in EDD of peptide anions.\textsuperscript{29} However, anion ETD resulted in extensive phosphate loss.

More recently, McLuckey and colleagues\textsuperscript{34} demonstrated sequential gas-phase ion--ion reactions to combine charge inversion and fragmentation by ETD. A phosphopeptide anion, generated by direct infusion of 1 $\mu M$ $\alpha$-casein tryptic digest in negative ion mode ESI, was charge-inverted to its doubly protonated form through reaction with highly protonated dendrimer and subsequently subjected to ETD. Altering ion charge after ion formation and prior to structural characterization provides new options for phosphoproteomic analysis, particularly for multiply phosphorylated peptides that are often absent from positive ion mode mass spectra.

Here, we utilize metal oxide-based enrichment of phosphopeptides to improve generation of multiply protonated species for both singly and multiply phosphorylated peptides, thereby allowing ECD of even a quadruply phosphorylated tryptic peptide. We also present the first demonstration of EDD of several serine-phosphorylated peptides from proteolytic digests and compare EDD to ECD.

**Experimental Section**

**Metal Oxide-Based Enrichment of Phosphorylated Peptides by $ZrO_2$ and $TiO_2$.** Lyophilized $\alpha$-casein and $\beta$-casein (Sigma, St. Louis, MO) were digested with either modified trypsin (Promega, Madison, WI) or endoprotease Glu-C (Roche, Boehringer Mannheim, Germany). Trypsin digestion was performed overnight at an enzyme to protein ratio of 1:50 in a 25 mM ammonium bicarbonate (Fisher, Pittsburgh, PA) solution at 37 °C. Glu-C digestion was performed overnight in 25 mM ammonium bicarbonate buffer at an enzyme to protein ratio of 1:100 at 25 °C. Digestion was quenched with formic acid, and resulting peptides were divided into 100 pmoles aliquots. Phosphorylated peptides were isolated from digestion mixtures via $TiO_2$- or $ZrO_2$-based enrichment according to our previously published protocol.\textsuperscript{18} Micropipette tips (Glygen, Columbia, MD) containing 50 $\mu g$ of $ZrO_2$ or $TiO_2$ microparticles were used for these experiments. Prior to phosphopeptide binding, metal oxide particles were equilibrated with a binding solution of 3.3% formic acid. Phosphopeptides were bound onto metal oxides; nonspecifically bound peptides were washed with HPLC grade water (Fisher Scientific, Fair Lawn, NJ), and phosphopeptides were eluted with a 0.5% piperidine solution. Collected phosphopeptides were dried in a vacuum concentrator (Eppendorf, Hamburg, Germany). Prior to mass spectrometric analysis, phosphopeptides were redissolved in either a water/acetonitrile (ACN)/2-propanol (IPA) (2:1:1) mixture with 0.25% piperidine for negative ion mode ESI or a water/ACN (1:1) mixture containing 0.1% formic acid for positive ion mode ESI. HPLC grade ACN and IPA were purchased from Fisher Scientific. Spin columns (Pierce Biotechnology, Rockford, IL) filled with 3 $\mu m$ $ZrO_2$ particles (ZirChrom Separations, Anoka, MN) were prepared in-house and used for sample volumes larger than 100 $\mu L$ under the same conditions described above for metal oxide microtip enrichment.

**Electron Detachment Dissociation and Electron Capture Dissociation Mass Spectrometry.** All spectra were acquired with a 7 T quadrupole(Q)-FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA), equipped with an Apollo I or Apollo II electrospray ion source. Phosphopeptide samples were
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ionized at a flow rate of 50–70 µL/h, mass selectively isolated with the quadrupole for MS/MS experiments, externally accumulated in a hexapole, and transferred to the ICR cell where they were captured by dynamic trapping. Two cell fills were used to improve ion signal-to-noise (S/N) ratios.

EDD was performed by irradiating trapped anions with a beam of ~20 eV electrons, generated from an indirectly heated hollow dispenser cathode (Heat Wave, Watsonville, CA) located at the rear side of the ICR cell, for 0.2–2 s. The electron irradiation time was tuned for optimized fragmentation efficiency for each target ion. For ECD, 0.5–1 eV electrons were reacted for 20–100 ms with multiply protonated phosphopeptides. In some experiments, activated ion (AI)-ECD\textsuperscript{35} and EDD were performed by employing infrared laser activation with limited radiation power and duration (5–7.5 W, 20–40 ms) prior to EDD and ECD.

All data were acquired with the Bruker XMASS program and processed with MIDAS FTMS analysis software,\textsuperscript{36} including Hanning apodization and one zero fill.

Results and Discussion

ECD and EDD of Singly Phosphorylated Peptides. Phosphorylated peptides from a trypsin digest of 100 pmol of β-casein were selectively isolated from nonphosphorylated peptides by employing ZrO\textsubscript{2}-based enrichment\textsuperscript{18} prior to mass spectrometric analysis. The final concentration of isolated peptides in the ESI solution was approximately 1.5 µM, estimated from the amount of protein used for digestion. ZrO\textsubscript{2} enrichment was applied for singly phosphorylated peptide enrichment because we have observed more selective isolation of singly phosphorylated peptides with ZrO\textsubscript{2} versus TiO\textsubscript{2} when employing negative ion mode ESI-MS for detection.\textsuperscript{18} However, in positive ion mode ESI, similar enrichment efficiencies were seen.\textsuperscript{18} The singly phosphorylated peptide generated from trypsin digestion of β-casein (β-casein:48–63, H-FQpSEEQQQT-

Figure 1a shows an ECD spectrum (64 scans, 40 ms irradiation at −0.8 V cathode bias) of the doubly protonated singly phosphorylated peptide ion, \([\text{M} + 2\text{H}]^{2+}\), at \(m/z\) 1031.42. Precursor ions were quadrupole isolated with an \(m/z\) window of 10 and accumulated in the external hexapole for 3 s. Only two c-type ions and two z-type ions from four of 15 backbone cleavages are observed, although phosphorylation was retained in all product ions that include the modified amino acid. However, the precise location of the modification site could not be determined due to the low sequence coverage. Similar results have been reported by Marshall and co-workers for protein kinase A phosphopeptides.\textsuperscript{37} The lack of backbone cleavage was explained by strong intramolecular interactions, e.g., a salt bridge, preventing product ion pairs from separating in ECD.

Figure 1b shows the ECD (64 scans, 20 ms irradiation at −0.8 V cathode bias) spectrum of the triply protonated form of the same peptide at \(m/z\) 687.95, observed following ZrO\textsubscript{2} enrichment.

Figure 1c shows an ECD (64 scans) of a doubly protonated singly phosphorylated peptide from trypsin digestion of β-casein. This charge state is dominant (for this peptide) in positive ion mode ESI prior to phosphopeptide enrichment. \(\gamma\) is a peak present prior to ECD. (b) ECD (64 scans) of the triply protonated form of the same peptide as in panel a. This charge state dominates in positive ion mode ESI-FT-ICR MS spectra of a nonenriched tryptic digest; however, this ion was masked by other abundant closely adjacent peaks. Only a very low or no signal was observed for the triply protonated form of the peptide without enrichment.

Precursor ions were mass selectively externally accumulated (10 m/z window) for 1 s. ECD of \([\text{M} + 3\text{H}]^{3+}\) yielded series of c-type ions and z-type ions with retained phosphorylation from cleavages at 12 of 15 backbone bonds, as summarized in the inset. The series of complementary c- and z-type ion pairs provides the nearly complete peptide sequence and the exact location of the phosphorylation at Ser50. ECD fragmentation efficiency was dramatically increased for the 3+...
charge state as compared to the 2+ charge state, consistent with the previously proposed increase in electron capture cross section with an increase in charge state, and with increased intramolecular Coulomb repulsion, facilitating product ion pair separation. Thus, accumulation of precursor ions with a higher charge state seems highly favored for ECD. Notably, ECD of the 3+ charge state was only achieved following metal oxide-based phosphopeptide isolation due to its extremely low abundance prior to isolation (a consequence of the acidity of this peptide and charge competition from less acidic species).

By contrast, acidic phosphorylated peptides are generally selectively detected in negative ion mode ESI. The same singly phosphorylated peptide was easily observed in negative ion mode ESI-FT-ICR MS in both its doubly and triply deprotonated forms, even without phosphopeptide isolation. The signal abundance of the 2− charge state was significantly increased after metal oxide enrichment. Improved signal abundance could also be obtained by increasing the hexapole ion accumulation time. However, that approach renders the analysis slow, particularly when considering the low fragmentation efficiency of EDD and the necessity of signal averaging. EDD (32 scans, 400 ms irradiation at −18 V cathode bias) of these precursor ions, [M − 2H]2−, is shown in Figure 1c. Precursor ions were mass selectively (10 m/z window) externally accumulated for 5 s. Several characteristic a− and x-type ions were generated from Cα−Cα backbone bond cleavages, although product ion abundances are low. EDD of [M + 2H]2−, cleaved nine of 15 backbone bonds without cleaving the phosphate group from Ser50, as illustrated in the inset of Figure 3a. Precursor ions were mass selectively externally accumulated for 3 s. Only two x-type ions with both phosphorylation is indicated by lowercase letters. Spectra were averaged over 32 scans. Peaks labeled with their m/z values correspond to unidentified fragments.

Figure 2a shows the ECD spectrum of a triply protonated singly phosphorylated peptide from a Glu-C digest of α-casein S1 isoform (α-casein-S1:126–140, monoisotopic mass = 1818.8335); 100 pmol of the digest was enriched for phosphorylated peptides with ZrO2, and isolated peptides were electrosprayed at a final concentration of 2 µM from an acidic solution for positive ion mode ESI-MS. The dominant charge state of this peptide was 2+ without enrichment, although the 3+ charge state was also observed. ECD of the triply protonated precursor ion, [M + 3H]3+ at m/z 607.29, resulted in cleavages at 11 of 13 cleavable backbone bonds (excluding the N-terminal side of Pro128, a cleavage that is only rarely observed in ECD). This spectrum unambiguously reveals Ser130 as the site of phosphorylation. Furthermore, extensive sequence information is obtained. EDD of the peptide in its most dominant charge state, [M − 2H]2− at m/z 908.41, is shown in Figure 2b, including observed cleavage sites in the peptide sequence. EDD disrupted only three backbone bonds in the peptide, resulting in few product ions with low abundance. However, as seen above for the β-casein singly phosphorylated peptide, the x12 product ion from EDD provides complementary information compared to ECD. Therefore, nearly complete sequencing and precise determination of Ser130 as the site of phosphorylation were accomplished by combining information from both ECD and EDD of this peptide.

ECD and EDD of a Doubly Phosphorylated Peptide. Peptides with multiple phosphorylations have ionization efficiencies much lower than those of their nonphosphorylated counterparts in positive ion mode; thus, such species are often only detectable in negative mode ESI-MS without application of prior isolation. A doubly phosphorylated peptide from trypsin digestion of the α-casein S1 isoform (α-casein-S1:58–73, DIG15,SE14,STEDQ,AMEDIK, monoisotopic mass = 1926.6842) was observed in a negative mode ESI-FT-ICR mass spectrum as the most dominant peak following TiO2 enrichment. EDD (16 scans, 0.5 s irradiation at −21 V cathode bias) was performed with the doubly deprotonated form of this doubly phosphorylated peptide, [M − 2H]2− at m/z 962.34, as shown in Figure 3a. Precursor ions were mass selectively externally accumulated (10 m/z window) for 3 s. Only two x-type ions with both phosphorylations retained were generated from two of 15 Cα−Cα bond cleavages. The precise locations of the two phosphate groups could not be identified because of the low sequence coverage.

The same doubly phosphorylated peptide was observed in its doubly protonated form in positive ion mode ESI-FT-ICR MS only after metal oxide isolation, as shown in the inset of Figure 3b. Figure 3b also contains the ECD spectrum from these precursor ions, [M + 2H]2+ at m/z 964.32, isolated from trypsin digestion of 100 pmol of α-casein followed by ZrO2 enrichment.
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Activated ion (AI)-ECD was performed by employing IR laser irradiation for 40 ms at 20% power (5 W). The laser fluence was carefully tuned to provide as much thermal activation as possible without causing fragmentation prior to ECD (25 ms irradiation at a cathode bias voltage of −0.8 V). Nearly complete sequence coverage was obtained, and the precise location of both phosphorylation sites at Ser61 and Ser63 was determined such species. Metal oxide enrichment with an in-house-ME enrichment with TiO$_2$ (chosen for its higher selectivity for multiply phosphorylated peptides) was performed prior to ESI-MS to improve the signal abundance of this peptide. EDD was performed for all charge states. Figure 4a shows EDD (eight scans, 0.6 s irradiation at −21 V cathode bias) of [M − 3H]$^-$ at $m/z$ 1039.41. Dominant neutral losses, corresponding to CO$_2$ and H$_3$PO$_4$, are observed from the charge-reduced, [M − 3H]$^-$, radical ion, but no product ions from backbone cleavages are seen. EDD of other charge states resulted in similar neutral losses with no evidence of peptide backbone cleavage. Other MS/MS fragmentation techniques, including CAD and infrared multiphoton dissociation (IRMPD), were therefore attempted in negative ion mode. However, only dominant sequential H$_2$PO$_4$ losses were observed, not providing any sequence information (data not shown). A shorter overlapping peptide with the protein sequence of residues 21–36 and four phosphorylations (β-casein: 21–36, LNVPGIEVEpSlpSpSPEE, monoisotopic mass = 2007.6804) generated from Glu-C digestion of β-casein was also observed in negative mode ESI-FT-ICR MS and subjected to EDD. However, similar neutral losses corresponding to CO$_2$ and H$_2$PO$_4$ were seen without backbone fragmentation.

We speculate that the limited fragmentation observed via EDD of quadruply phosphorylated peptides is in part due to the gas-phase peptide structure(s). Highly phosphorylated peptides may be more prone to forming intramolecular salt bridges, and there is a possibility that zwitterionic structures are involved. Another possibility is a decreased EDD fragmentation efficiency with an increase in mass.

The lack of sequence information from all attempted negative ion mode MS/MS strategies for the quadruply phosphorylated peptide demanded other approaches to characterizing such species. Metal oxide enrichment with an in-house-prepared spin column was employed in selectively isolating phosphorylated peptides from trypsin digestion of a mixture of α-casein and β-casein. Isolated phosphopeptides at a final concentration of ~1.2 µM in a spray solution for positive ion mode ESI were introduced into a dual-stage ion funnel source, recently implemented with our instrument. The quadruply phosphorylated peptide (β-casein:16–40, RELEELNVPGIEVEpSlpSpSPEE, monoisotopic mass = 3121.2582) was observed, mostly in its triply protonated form, [M + 3H]$^{3+}$ at $m/z$ 3121.2582) from one trypsin missed cleavage, containing four phosphorylations at Ser30, Ser32, Ser33, and Ser34. The latter peptide has a pI as low as 2.5 as well as four phosphorylations and only two arginines (Arg).

![Figure 3. (a) EDD of a doubly deprotonated doubly phosphorylated peptide from trypsin digestion of β-casein. (b) ECD of the doubly protonated form of the same peptide. The inset shows that this ion, at $m/z$ 962.34, is observed only following phosphopeptide enrichment.](image-url)

EDD and EDD of Quadruply Phosphorylated Peptides.

Tryptic digestion of β-casein generally produces two phosphopeptides: the 48–63 peptide discussed above, containing one phosphorylation on Ser50, and the protein sequence of residues 16–40 (β-casein:16–40, RELEELNVPGIEVEpSlpSpSPEE, monoisotopic mass = 3121.2582) from one trypsin missed cleavage, containing four phosphorylations at Ser30, Ser32, Ser33, and Ser34. The latter peptide has a pI as low as 2.5 as calculated by the ScanSite pI/Mw program accessible at Exasy Tools (www.expasy.org), due to its seven glutamic acids (Glu) as well as four phosphorylations and only two arginines (Arg). Thus, it is not surprising that the peptide was not detected in positive ion mode ESI-FT-ICR MS without enrichment. A related quadruply phosphorylated peptide (β-casein:16–43, RELEELNVPGIEVEpSlpSpSPEE, monoisotopic mass = 3476.4803) has been detected via Lys-C digestion, which adds three amino acids at the C-terminus (including one lysine (Lys43)), followed by LC positive ion mode ESI-MS.

We began to characterize peptide 16–40 in negative ion mode by utilizing EDD and other fragmentation techniques involving thermal activation to investigate the obtainable analytical information (peptide sequence and sites of phosphorylation). The peptide was observed with charge states ranging from 2− to 6−, with 3− at $m/z$ 1039.41 being dominant in negative ion mode ESI-FT-ICR MS. Metal oxide enrichment with TiO$_2$ (chosen for its higher selectivity for multiply phosphorylated peptides) was performed prior to ESI-MS to improve the signal abundance of this peptide. EDD was performed for all charge states. Figure 4a shows EDD (eight scans, 0.6 s irradiation at −21 V cathode bias) of [M − 3H]$^-$ at $m/z$ 1039.41. Dominant neutral losses, corresponding to CO$_2$ and H$_3$PO$_4$, are observed from the charge-reduced, [M − 3H]$^-$, radical ion, but no product ions from backbone cleavages are seen. EDD of other charge states resulted in similar neutral losses with no evidence of peptide backbone cleavage. Other MS/MS fragmentation techniques, including CAD and infrared multiphoton dissociation (IRMPD), were therefore attempted in negative ion mode. However, only dominant sequential H$_2$PO$_4$ losses were observed, not providing any sequence information (data not shown). A shorter overlapping peptide with the protein sequence of residues 21–36 and four phosphorylations (β-casein: 21–36, LNVPGIEVEpSlpSpSPEE, monoisotopic mass = 2007.6804) generated from Glu-C digestion of β-casein was also observed in negative mode ESI-FT-ICR MS and subjected to EDD. However, similar neutral losses corresponding to CO$_2$ and H$_2$PO$_4$ were seen without backbone fragmentation.

We speculate that the limited fragmentation observed via EDD of quadruply phosphorylated peptides is in part due to the gas-phase peptide structure(s). Highly phosphorylated peptides may be more prone to forming intramolecular salt bridges, and there is a possibility that zwitterionic structures are involved. Another possibility is a decreased EDD fragmentation efficiency with an increase in mass.
the locations of all four sites. IRMPD (10 W, 80 ms) of the same precursor ions at \( m/z 1041.43 \), shown in Figure 4c, generated b/y ions from 17 amide bond cleavages, surprisingly with nearly all phosphorylations retained. Proline-rich and acidic amino acid-rich phosphopeptides have been observed to produce b- and y-type ions retaining labile phosphate groups in CAD. Preferred backbone amide bond cleavages N-terminal to proline and C-terminal to glutamic acid in vibrational activation methods such as IRMPD may efficiently compete with cleavages at phosphate groups, thus producing backbone fragments retaining phosphorylations.

Conclusions

We have demonstrated the utility of metal oxide-based enrichment of phosphopeptides in improving sequence coverage and allowing unambiguous determination of the locations of phosphorylations by the gas-phase ion-electron reactions ECD and EDD. The increased signal and increased average charge state of phosphorylated peptides in positive ion mode after selective isolation by metal oxides allowed application of ECD, which resulted in extensive backbone fragmentation, thereby improving the quality and extent of proteomic information. Furthermore, metal oxide-based phosphopeptide enrichment allowed highly acidic peptides with multiple phosphorylations to be detected in positive ion mode and subjected to ECD. For the first time, ECD of the quadruply phosphorylated peptide from trypsin digestion of \( \beta \)-casein was demonstrated. EDD provided very limited sequence information because of its low fragmentation efficiency. However, complementary information was observed in EDD of singly phosphorylated peptides compared to ECD. The facile detection of acidic phosphopeptides in negative ion mode may still render EDD a valuable alternative method for phosphoprotein analysis.

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References

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