HHS Public Access

Author manuscript

J Proteome Res. Author manuscript; available in PMC 2018 February 03.

Published in final edited form as:

J Proteome Res. 2017 February 03; 16(2): 1069–1076. doi:10.1021/acs.jproteome.6b00487.

Neutral Loss is a Very Common Occurrence in Phosphotyrosine-containing Peptides Labeled with Isobaric Tags

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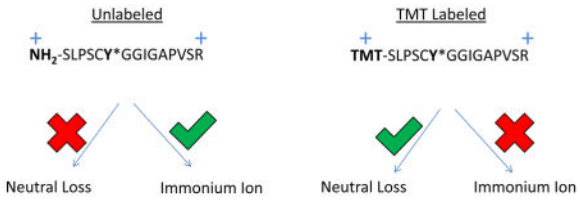
Abstract

While developing a multiplexed phosphotyrosine peptide quantification assay, an unexpected observation was made – significant neutral loss from phosphotyrosine (pY) containing peptides. Using a 2000-member peptide library, we sought to systematically investigate this observation by comparing unlabeled peptides with the two highest-plex isobaric tags (iTRAQ8 and TMT10) across CID, HCD and ETD fragmentation using high resolution high mass accuracy Orbitrap instrumentation. We found pY peptide neutral loss behavior was consistent with reduced proton mobility, and does not occur during ETD. The site of protonation at the peptide N-terminus changes from a primary to a tertiary amine as a result of TMT labeling which would increase the gas phase basicity and reduce proton mobility at this site. This change in fragmentation behavior has implications during instrument method development and interpretation of MS/MS spectra, and therefore ensuing follow-up studies. We show how sites not localized to tyrosine by search and site localization algorithms can be confidently re-assigned to tyrosine using neutral loss and phosphotyrosine immonium ions. We believe these findings will be of general interest to those studying pY signal transduction using isobaric tags.

Graphical Abstract



Keywords

Tandem Mass Tag (TMT); isobaric tag; phosphotyrosine; neutral loss; proton mobility; phosphorylation; immonium ion; high-energy collision-induced dissociation (HCD); site localization

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Introduction

Signal transduction through tyrosine phosphorylation triggers numerous cellular processes including adhesion, proliferation and immune response[1–2]. Since aberrant regulation of tyrosine phosphorylation has been implicated in many diseases[3], their study is of increasing importance. While phosphorylated peptides require enrichment to separate them from non-phosphorylated background peptides for efficient detection[4], tyrosine phosphorylation is especially rare, accounting for less than 1 % of the phosphoproteome[5]. The low abundance of these regulatory PTMs typically requires immunopurification prior to analysis by mass spectrometry[6]. In addition to being much less frequent than phosphoserine and phosphothreonine peptides, phosphotyrosine peptides also have some unique structural characteristics.

While phosphoserine and phosphothreonine peptides are well known for producing a dominant neutral loss of phosphoric acid during collision-induced dissociation (CID) and to a lesser extent in high-energy collision-induced dissociation (HCD)/beam-type fragmentation, neutral loss for phosphotyrosine peptides is generally considered to be a rare event in positive ion mode[7–11]. This is in contrast to negative ion mode where all three residues readily lose phosphate (−79)[12–13]. The C-O bond is stabilized by tyrosine's aromatic ring, preventing H_3PO_4 loss as in pS or pT [7] so the less favorable O-P bond must be cleaved for neutral loss of hydrogen phosphite (HPO_3). Unlike phosphoserine and phosphothreonine, bond cleavage around phosphotyrosine is comparable for both electron transfer dissociation (ETD), which does not cleave phosphate moieties, and CID[14]. Phosphotyrosine peptides can even retain the phosphate moiety upon internal fragmentation of the peptide, yielding a unique phosphotyrosine immonium ion at 216.0426 *m/z* [15].

Isobaric tagging can be particularly useful for low abundance PTM studies that may benefit from combining the signal across all channels to give brighter precursor intensity in the MS^1 scan and more intense structural ions in the MS^2 scan, thus aiding both identification and site localization. Multiplexing without making the MS^1 spectrum more complex is desirable – a 10-plex TMT run has a much less complex MS^1 spectrum than a three-plex MS^1 methodology such as triple label SILAC. In addition to an efficient use of instrument time, when replicates are included within an isobaric labeling experiment, day-to-day instrument performance as a source of variability between replicates is eliminated and missing peptides between different replicate analyses are also eliminated.

Previous combinations of phosphotyrosine immunopurification and isobaric tagging [16–18] yielded interesting biological insights, but have not focused on any unique fragmentation characteristics that govern netural loss and immonium ion formation upon dissociation of isobarically tagged phosphotyrosine peptides. The goal of this study was to systematically examine the fragmentation characteristics of isobarically labeled phosphotyrosine peptides using a synthetic library containing 2000 members. High resolution MS/MS data was obtained on the library examining: CID, HCD, and ETD on unlabeled, TMT labeled and iTRAQ8 labeled samples. In stark contrast to unlabeled phosphotyrosine peptides, labeled phosphotyrosine peptides readily form neutral loss during both CID and HCD. These findings can inform future studies examining the phosphotyrosine proteome using isobaric

tags and challenge phosphorylation site localization and search algorithms to include the phosphotyrosine immonium ion and neutral loss peaks.

Methods

A library of two thousand synthetic phosphotyrosine peptides with and without isobaric labeling was used for comparison. The library was comprised of 1000 8-mers of the sequence AG[LREA][FSTDG][phosphoY][PHDAFE][LREA][ST]K as well as 1000 15-mers of the sequence AG[LREA][FSTDG][phosphoY][PHDAFE][LREA][ST]HELPDGK. The library design was based on the sequence of a known pY peptide and then positions were substituted on both sides of the pY site. The peptides were synthesized at Cell Signaling Technology (Danvers, MA).

TMT (Pierce, Rockford, IL) and iTRAQ8 (Sciex, Framingham, MA) labeling was performed in 100 mM HEPES pH 8.5 and 0.2 mg of isobaric tag was added per 0.1 mg of peptide and allowed to react for 1 hr. The reaction was quenched by adding hydroxylamine to a final concentration of 0.5%. Peptides were then purified using tC18 Sep-Pak Cartridges (Waters, Milford MA) and injected onto a Fusion Tribrid mass spectrometer[19] (Thermo-Fisher Scientific). 2 μg of the pY library was injected on to a 30 cm, 75 μm id column and peptides were separated using an Easy nLC 1000 (Thermo). The column was packed with C18 1.8 μm dp beads with 12 nm pores (Sepax Technologies Inc., Newark, DE) and was heated to 60°C using an in-house built column oven. A back-to-back high resolution (15k @ 200 m/z) CID/HCD MS^2 method employing 35% collision energy was used for the CID/HCD comparison. The high resolution (15k @ 200 m/z) ETD MS^2 method used calibrated charge-state specific reaction times[20] for z = 2–7 and included supplemental activation for z=2 peptides[21] to enhance product ion yield for that charge state, both standard features of ETD enabled Orbitrap Fusion instruments. The Sequest search engine[22] (version 28) was utilized to match MS/MS spectra to peptides at an FDR of 1% at the peptide level as measured via the target-decoy method [23].

To assess fragmentation properties of peptides, expected fragment ions were extracted from MS^2 spectra. In addition to the phosphotyrosine immonium ion (216.0426 Da), neutral losses of 79.96633 Da (HPO_3) and 97.97689 Da ($HPO_3 + H_2O$) were considered (± 0.02 Th) and their intensities were held relative to the base peak. Neutral losses were counted as significant if they were ≥ 1/3 of the base peak. The prevalence of phosphate neutral loss and phosphotyrosine immonium ion formation was assessed and correlated with fragmentation type, charge state, peptide sequence, and proton mobility. Proton mobility was evaluated by comparing the observed charge state with the numbers of basic sites (Arg, Lys, and His residues, as well as N-termini): a charge state exceeding the numbers of basic sites was taken to suggest high proton mobility, while a charge state less than or equal to the number of basic sites implied low proton mobility.

The breast cancer cell line MCF10A was cultured in DMEM:F12 supplemented with 5% horse serum, 20ng/mL EGF, 10μg/mL insulin, 0.5mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 1% penicillin/streptomycin. Cells were plated in 15-cm plates and grown in a 5% CO_2 incubator at 37°C. At 80% confluency, cells were washed twice with cold PBS

and incubated for 18hr in starve medium before growth factor stimulation. Cells were stimulated with 20ng/mL EGF at 30 seconds, 2 minutes (min), 5 min, 7 min, 10 min, 15 min, 30 min, and 60 min or left untreated (represented as time point 0 min). One additional 15-cm plate represents basal MCF10A growth (no starvation) to serve as a control. In total, ten 15-cm plates each represent one condition for a total of 10 cell pellets. The cell pellets were immediately snap frozen in liquid nitrogen and stored at −80°C.

Cell pellets were lysed in 1mL lysis buffer (2% SDS, 150mM NaCl, 50mM Tris, Roche complete protease inhibitors, phosphatase inhibitors, and 2mM sodium orthovanadate). Samples were reduced with 5mM dithiothreitol (DTT) and alkylated with 14mM iodoacetamide for 45min at room temperature in the dark. Samples were quenched with 5mM DTT and methanol/chloroform precipitated. Proteins were denatured and sonicated in 1mL 8M urea and 25mM HEPES (pH 8.5). Proteins were quantified using a bicinchoninic (BCA) assay. The urea concentration was diluted to 4M to digest proteins (~1.2mg per sample) with Lys-C (enzyme-to-protein ratio of 1:500) for 2 hours at 37°C while shaking. The concentration of urea was diluted further to 2M and digested overnight with both Lys-C (1:500) and trypsin (1:100) at 37°C. The following morning, a final aliquot of trypsin (1:100) was added after dilution to 1M urea for a 6 hr incubation while shaking at 37°C. Samples were acidified with formic acid to pH 3. A quality control check was performed to determine the miscleavage rate (3–4%) for two different samples prior to clean up with a 100mg SepPak (Waters) column. The SepPak eluents were dried using a vacuum centrifuge.

Peptides were subjected to phosphopeptide enrichment using TiO_2 (GL Sciences). Peptides were dissolved in 50% acetonitrile (ACN) and 2M lactic acid (LA) and vortexed with TiO_2 beads (1:4 peptides-to-beads) for 1.5 hrs at room temperature. Three washes were performed in the order of 50%ACN/2M LA, 50%ACN/0.1% triflouroacetic acid (TFA), and 25%ACN/0.1%TFA. Two sequential phosphopeptide elutions were performed with 200μL 50mM HK_2PO_4 (pH 10) for 10min while vortexing at room temperature. Phosphopeptides were acidified to 1% formic acid and desalted with C18 StageTip prior to isobaric labelling with TMT reagents. Phosphopeptides were resuspended in 200mM EPPS (pH 8.5) and quantified with the BCA assay. Approximately 15μg of phosphopeptides (per channel) were labelled with 3μL of 20μg/μL of the corresponding TMT 10-plex reagent (Thermo) for 1.5 hr at room temperature. The reaction was quenched with 5% hydroxylamine for 15 min at room temperature. The ten samples were mixed using the normalized TMT labelling ratios, acidified and desalted using a 10mg SepPak. The SepPak eluent was dried using a vacuum centrifuge overnight.

Phosphopeptides were resuspended in 1mL IAP buffer (50mM MOPS/NaOH pH 7.2, 10mM Na_2HOP_4, 50mM NaCl). The phospho-tyrosine monoclonal antibody P-Tyr-1000 (100μg; Cell Signaling Technology) was coupled to 30μL protein A agarose beads (Roche) overnight at 4°C with head-over-end rotation. The antibody resin was washed 4 times with 1mL cold PBS. The TMT-labelled phosphopeptides were added to the immobilized antibody slurry and incubated for 2 hrs at 4°C with head-over-end rotation. The immobilized antibody beads were washed twice with 1mL IAP buffer followed by 1mL cold H_2O at 4°C. Phosphotyrosine peptides were eluted from the conjugated beads twice with 75μL 100mM formic acid for 10min at room temperature. Phosphopeptides were desalted on a C18

StageTip prior to LC-MS2-MS3 analysis using a 3hr gradient and allowing multiple charge-states per precursor to be sequenced on the aforementioned liquid chromatography/mass spectrometry platform.

Figures S-1, S-2, S-3, S-4a-f and Tables S-1, S-2 and S-3 can be found in Supporting Information.

Results and Discussion

During an effort to develop a multiplexed phosphotyrosine analysis protocol, it was observed that large numbers of phosphotyrosine-containing peptides when prepared with TMT labels unexpectedly showed neutral loss of HPO_3. An example is shown in Figure 1. As expected, manual inspection of an unlabeled peptide showed no noticeable neutral loss of phosphate (Figure 1, Top). However, upon TMT labeling, the MS/MS spectrum of the same peptide at the same charge state exhibited not only noticeable neutral loss, but the neutral loss was the base peak of the spectrum (Figure 1, Bottom). To determine if this was a special case or a more general phenomenon, a library containing 2000 members was synthesized to systematically investigate neutral loss formation under different fragmentation mechanisms and with two different isobaric tags, TMT and iTRAQ8. All library analyses were conducted using high resolution accurate mass instrumentation to accurately determine mass and charge state for both precursor and product ions. This prevented contamination from pseudo-neutral loss peaks, which are indistinguishable on a low resolution instrument[24]. Moving beyond the single example discussed previously, Figure 2 shows that TMT labeling clearly enhances neutral loss of phosphotyrosine peptides, giving a 5X increase in the number of peptides having a phospho-neutral loss ≥ 1/3 of the base peak. The '≥ 1/3 of the base peak' rule was implemented as a rough filter to remove from consideration spectra with very low neutral loss intensity, which may include noise ions. Furthermore, using a relative intensity controlled for variation in abundance across pY-containing peptides. The rule was uniformly applied regardless of fragmentation mechanism or isobaric tag type. For the library analyses, neutral losses of 79.96633 Th (HPO_3) and 97.97689 Th ($HPO_3 + H_2O$) were considered (±0.02 Th).

To further investigate the observed increase in HPO_3 neutral loss upon isobaric tagging, comparisons were made between the iTRAQ8 and TMT reagents, as these represent the highest commercially available multiplexing capabilities of 8-plex and 10-plex, respectively. Multiple fragmentation mechanisms were compared as well, including CID, HCD, and ETD. The goal was to determine if the neutral loss formation was specific to either TMT or CID. Figure 2 shows that peptides bearing either isobaric tag yield significant neutral losses at similar rates, with the iTRAQ8 reagent slightly favoring neutral losses at the individual charge state level. The column depicting "All" identified phosphopeptides shows a higher degree of similarity between the two tags than the individual charge state data because the charge state distribution of identified phosphopeptides was higher for iTRAQ8 than TMT as previously reported[25]. This resulted in the TMT results having a higher percentage of $z = 2$ (often high neutral loss) peptides identified than in the iTRAQ8 data. The high overall similarity in neutral loss percentage ruled out any TMT-specific effects, implying that increased neutral losses were instead a general property shared by both isobaric tagging

reagents. However, the structure of the iTRAQ8 reagent has not been released, and since the higher multiplexing capacity of the 10-plex TMT can increase the scope and sensitivity of quantitative proteomics, all subsequent experiments focused on the TMT reagent.

As CID is considered a slow heating process[26], the reaction kinetics are such that upon dissociation, the phosphate group can actually move from its native position to another amino acid residue on the same peptide i.e., a gas-phase intramolecular transfer of phosphate [27–28]. To determine whether the observed neutral losses could actually reflect isobaric tag-enhanced scrambling of the phosphate moiety to a nearby serine or threonine, an alternative fragmentation mechanism, higher-energy collision dissociation (HCD), was examined. Figure 2 depicts that neutral loss of HPO_3 has a strong occurrence in HCD (more than 3X higher than in the unlabeled sample), albeit to a lesser extent (24%) compared to CID (37% for TMT labeled peptides). This is expected as HCD imparts high energy rapidly and incorporates secondary collisions that reduce neutral loss peak intensity. Importantly, gas-phase rearrangement of phosphate does not occur during the microsecond-scale activation time of beam-type fragmentation e.g., HCD [27], eliminating this as the cause of enhanced neutral loss formation. To further address the potential of isobaric tag-enhanced phosphate scrambling from Y to an S or T, Figure S-1 depicts CID fragmentation of the peptide IYNGDY*YR (where * denotes phosphorylation) producing large neutral loss peaks (>40% relative to the base-peak), despite not having an S or T in its sequence.

For unlabeled phosphopeptides, it was shown by Coon et al.[29] that no neutral loss is observed during electron transfer dissociation (ETD). A comparison was made to determine if TMT labeling enhanced neutral loss during radical-driven fragmentation on the Orbitrap Fusion, which uses Townsend discharge ionization as opposed to a filament to create radical fluoranthene anions. In no case was phosphotyrosine neutral loss observed (Figure 2) leading to the conclusion that TMT does not enhance pY neutral loss during ETD.

Since radical-driven fragmentation did not display altered neutral loss, the focus returned to CID/HCD where the mobile proton model is used to explain fragmentation[30]. When a mobile proton is available, charge-directed backbone fragmentation can occur more readily and fragments from the neutral loss (charge remote) fragmentation pathway are less frequently observed. Likewise, neutral loss pathways become more prominent in the absence of mobile protons. For the purpose of this work, peptides were separated into two classes – low and high proton mobility. The low proton mobility state was defined as when the charge state was ≤ the number of basic sites that peptide contained. Basic sites referred to lysine, arginine, histidine and the peptide N-terminus. A peptide with high proton mobility would therefore have a charge state that is greater than the number of basic (protonatable) sites. Any proton not ‘held’ by basic sites is free to mobilize across the peptide backbone facilitating charge-directed amide bond cleavage.

Proton mobility can thus be manipulated by either changing the number of basic sites (the peptide sequence), or the peptide charge state. Since the dramatic increase in phosphotyrosine neutral loss upon isobaric labeling occurred during comparison of the same library, the peptide sequences and therefore the number of basic sites did not change. One way TMT labeling could reduce proton mobility would be if a dramatic decrease in the

charge state distribution occurred. But Figure S-2 (left panel) shows that TMT labeling does not dramatically decrease the charge state distribution, and in fact causes a slight increase from an average charge state of 2.56 before TMT labeling to 2.93 after labeling. This slight increase has also been observed by Thingholm et al.[25]. The increase in charge elevates the number of observed mobile protons from an average of −0.93 mobile protons before labeling to −0.33 after (Figure S-2 right panel). Since the peptide sequences remained constant and the change in average charge state was slight, how might TMT labeling be reducing proton mobility allowing charge remote fragmentation pathways to dominate? Studying the reaction chemistry of TMT labeling (Figure 3) indicates that TMT labeling replaces a primary amine for a tertiary amine as the N-terminal protonation site. Thus, TMT labeling doesn't change the number of basic residues, but rather the *type* of basic residues. In the gas phase, the rank order for the basicity of amines is: tertiary > secondary > primary [31]. Gas phase basicity (GB) is directly proportional to proton affinity (PA) as seen in the equation: $GB = PA - T\Delta S$ [32] resulting in protons being more tightly held (less mobile) on the tertiary amine of the TMT tag. Proton mobility was a significant factor in predicting neutral loss formation (two-sided p-value $2.45e^{-143}$, Table S-1) with 97% of the peptides giving a neutral loss having low proton mobility. Due to the increased gas phase basicity of the TMT tag, the mobility of protons is lower on a TMT labeled peptide than an unlabeled peptide of the same sequence and charge state. One noteworthy exception was found to occur if the peptide had a proline (P). Since bond cleavage is often enhanced at proline [33], this has impacts on neutral loss formation. An overall reduction in neutral loss was seen in peptides containing a proline (Table S-2). A similar reduction has been reported in unlabeled phospho-serine and threonine peptides [34].

At first glance, one might think that neutral loss could be enhanced simply by adding an arginine or a histidine as the N-terminal residue, both of which provide amines of greater basicity than a primary amine. But the N-terminal primary amine would still remain, meaning that *two* basic sites would be added to the peptide by adding an arginine at the end, most likely altering the charge state. In contrast, TMT replaces a primary amine for a tertiary amine, keeping the number of basic sites constant. To further support the role of proton mobility in phosphotyrosine neutral loss, an examination of the few unlabeled peptides that yielded a noticeable neutral loss (Figure S-3) reveals that only in cases of extremely low proton mobility, having a high ratio of basic sites to detected charge, does neutral loss occur. The charge states of these peptides are likely highly unfavorable and was only observed here due to the high concentration of the library sample and would likely never be observed in a real-world sample (e.g., 4 basic sites on a peptide having z=2).

Understanding the fragmentation characteristics of phosphotyrosine peptides is useful for at least two reasons: 1. this knowledge enables higher confidence before doing labor and cost intensive follow up studies such as site directed mutagenesis and production of novel phosphosite-specific antibodies, and 2. for recovering sites that received a low localization score by site-scoring algorithms, particularly since credit is not given for neutral loss and immonium ions. Two characteristics that have been helpful for distinguishing phosphorylation on a tyrosine as opposed to a serine or threonine have been the phosphotyrosine immonium ion and the ratio of HPO_3 (80) to H_2PO_4 (98). The 98 loss is generally much stronger in the case of phospho-serine/threonine while 80 often

predominates in phosphotyrosine[7, 35]. Most often, the neutral loss for tyrosine is absent for unlabeled peptides and this absence is sometimes used as evidence of tyrosine phosphorylation. While phosphotyrosine does not lose phosphoric acid, a concomitant loss of water from another residue on the peptide can still yield a loss of 98. Neutral loss on phosphotyrosine peptides is so rare, that no correlation between neutral loss and immonium ion formation has been discussed. In the case of isobaric tagged peptides where neutral loss is common, it is important to note that by being a phospho-intact fragment ion, phosphotyrosine immonium ion formation is thus inversely proportional to neutral loss formation. HCD data in Figure 4A reveals that for isobarically labeled peptides, immonium ion formation is directly proportional to proton mobility. Peptides that had a histidine or arginine (protonated residues) ± two residues of the phosphorylation site were the exceptions. With a proton being so close to the site of phosphorylation, nearby backbone cleavage was enhanced. Interestingly, the immonium ion was never observed if a basic residue was left unprotonated (i.e., a negative number of mobile protons).

Moving beyond the library, TMT labeled phosphopeptides from a 10-point EGF stimulation time course of MCF10A cells using the CID MS2/HCD MS3 approach were examined. The goal was to see if there were examples of phosphorylation sites that were not localized to tyrosine by the search engine that could be assigned to tyrosine using observation of the neutral loss and phosphotyrosine immonium ions. Since phosphotyrosine peptides are often very low abundant, the quality of spectra is often lower, making localization more difficult. The pY enrichment protocol used here performs immunopurification on a previously phospho-enriched sample, so non-tyrosine phosphopeptides can be non-specifically pulled down and are typically seen in pY analyses (albeit at a lower % than pY peptides). Unlocalized peptides are either discarded or reported separately as they cannot be used for motif analysis and literature searches, and have reduced utility for follow up studies. About 10% of the phosphopsites were not assigned to tyrosine. Ten such examples are listed in supplementary Table S-3. In each case, the search engine assigned the site of phosphorylation to an S or T rather than a Y residue. The A-score algorithm[36] was then used to see if any localization changes could be made automatically. Three of the ten sites were re-assigned as phosphotyrosine sites with localization scores >13 (>95% confidence). The remaining peptides did not have passing A-scores, but could nevertheless be localized to tyrosine using neutral loss and immonium ions, which are not considered by the localization algorithm. Spectra for the peptides not localized by Ascore are shown in Figure S-4a–f. Two peptides for which localization at either S or Y could not be distinguished, showed a neutral loss of 80 (HPO_3) that was greater than or equal to the loss of 98. Previously it has been shown that serine will most likely exclusively lose 98[7, 35]. Since the HCD collision energy of 55 used during the MS3 scan is optimal for both reporter ion intensity and immonium ion formation, this data was found to be quite useful, especially when a single tyrosine is present in the peptide sequence. An example is shown in Figure 4B from the SHC1 peptide ELFDDPS*YVNVQNLDK, where the site was assigned to serine. The MS3 scan however, clearly shows the presence of the phosphotyrosine immonium ion. This site on SHC1, Y317 (or Y427 depending on the isoform), has been shown previously as an EGF induced phosphotyrosine[37]. Four of the non-localized peptides had multiple tyrosines, but *b* and *y* ions could be used to eliminate certain tyrosines such that only one Y was potentially the

site along with an S or T residue. In these cases, the immonium ion can be as diagnostic as is if a single Y was present. Sequence ions can also be observed in the MS3 scan and can occasionally be used for localization as well, arguing that the MS^3 scan has value beyond quantification.

To improve pY spectrum interpretation, it may be useful to try to augment immonium ion formation while diminishing neutral loss intensity. Linke et al., found that high collision energy (CE) is required during HCD to optimize pY immonium ion signal[38]. For quantitative accuracy, we use an MS^3 based approach using CID-MS^2 for identification and HCD-MS^3 at high collision energy for quantification[39–40]. Thus the MS^3 approach, in which the CE is optimized for reporter ion signal, is also optimized for pY immonium ion signal. When keeping the peptide sequence constant, increasing the charge state should also increase immonium ion and reduce neutral loss signal. One way to achieve this would be by adding supercharging reagents such as m-nitrobenzyl alcohol (m-NBA) to the mobile phase[41]. This addition improved phoshopeptide identification rates via ETD fragmentation, but also altered chromatographic performance[42], which could be overcome by co-spraying the supercharging reagent using a dual-spray source[43].

The occurrence of neutral loss and immonium ions could have implications on instrument method settings. For example, multi-stage activation which has shown improved identification rates for phospho-serine and phospho-threonine peptides[44] could be investigated for its utility for isobarically tagged phosphotyrosine peptides, but including the loss of 80 (HPO_3) which is not often included. As noted by Linke et al., optimal analysis of isobarically tagged phosphopeptides involves a balance between identification, localization and quantification[38]. They suggested a combined CID-MS^2/HCD-MS^2 approach similar to the combined CID-MS^2/HCD-MS^3 approach recommended here. The combination of multiple charge states per precursor provides additional evidence, so we allow multiple charge states per peptide to be sequenced during pY analyses. After stringent FDR filtering on all identified peptides, combining CID and HCD data from all measured charge states of a peptide into a ‘composite’ spectrum may yield optimal site localization results, but this approach is not currently available.

Adding neutral loss and immonium ions to search algorithms may enhance overall identification performance, but this option is rarely if ever used in standard search algorithms. Pichler et al., has noticed that isobaric tagged spectra can have additional ions not typically assigned by standard search algorithms[45]. Perhaps adding both loss of isobaric tag and phospho-specific ions (neutral loss and immonium ions) to search algorithms would yield optimal results. Since the neutral loss of 80 and the phosphotyrosine immonium ion are typically not given credit in site localization algorithms, knowing when and where to expect to see them can increase confidence during manual site localization assignment. In the case of low proton mobility, one should look for a neutral loss of 80 (although 98 may also be strong) and in cases of high proton mobility search for the immonium ion (in HCD spectra). Some exceptions may be observed if a proline is present or if the site of protonation is adjacent to the site of phosphorylation. Especially useful is the case where spectra for multiple charge states of the same peptide are collected and both pieces of evidence can be incorporated.

Conclusions

A library of 2000 phosphotyrosine peptides was used to conduct an in-depth examination of neutral loss formation post isobaric labeling using high resolution accurate mass data. The general rule that phosphotyrosine neutral loss is a rare event does not apply after isobaric tagging. The neutral loss from isobarically tagged peptides is inversely proportional to proton mobility and does not occur during ETD. One contributing factor is that TMT labeling replaces a primary amine for a tertiary amine, which increases gas-phase proton affinity, allowing protons to be more tightly held. As a result, proton mobility is lower on a TMT labeled peptide than an unlabeled peptide of the same sequence and charge state. Immonium ion formation was found to be inversely proportional to neutral loss formation. For isobaric tagged peptides, knowing when and where to look for HPO_3 loss and immonium ion formation may inform instrument method development and supplements site localization algorithms, enabling recovery of peptides with low site localization scores and aiding follow-up experiments e.g. site mutation and site-specific antibody creation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

R.A.E., A.R.E. and S.P.G. are partially funded through NIH grants P50-GM107618 and U54HL127365. SPG is partially funded through NIH grant GM67945.

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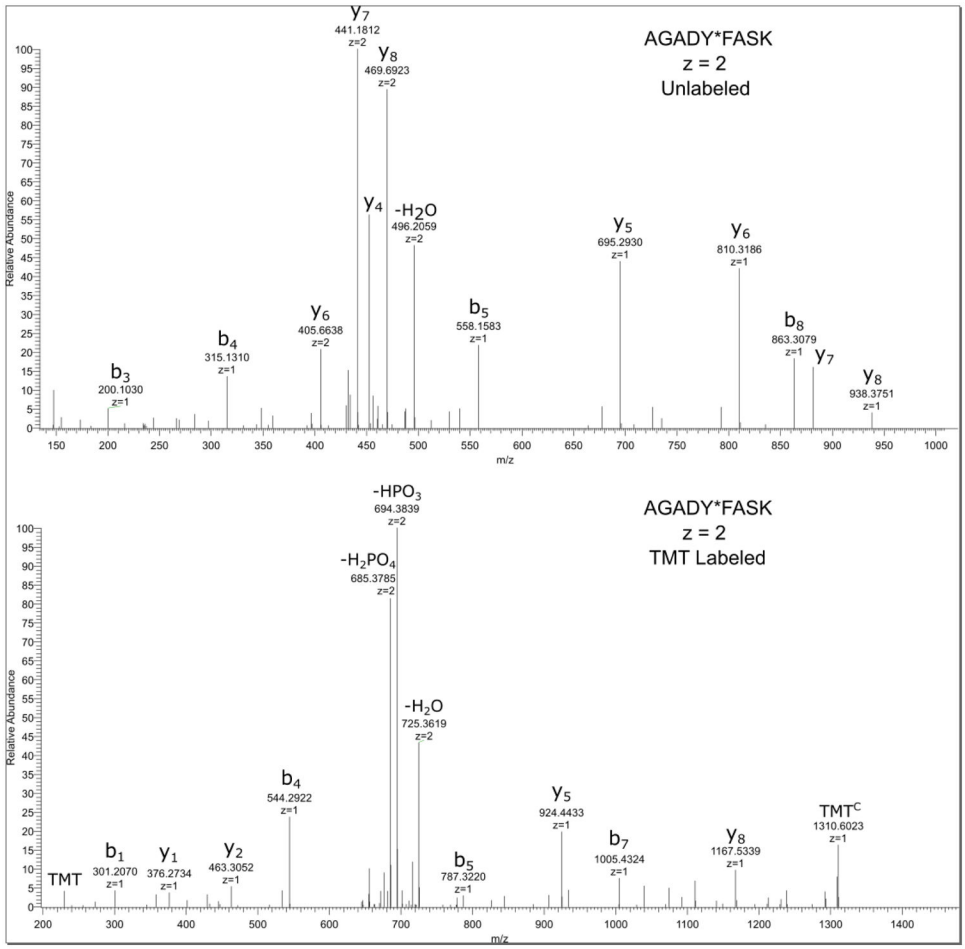

Figure 1.

CID MS^2 spectra comparing the same peptide at the same charge state in both unlabeled (top) and TMT labeled (bottom) forms. Prior to labeling no neutral loss is observed, but after labeling the neutral loss beak is the base peak of the spectrum.

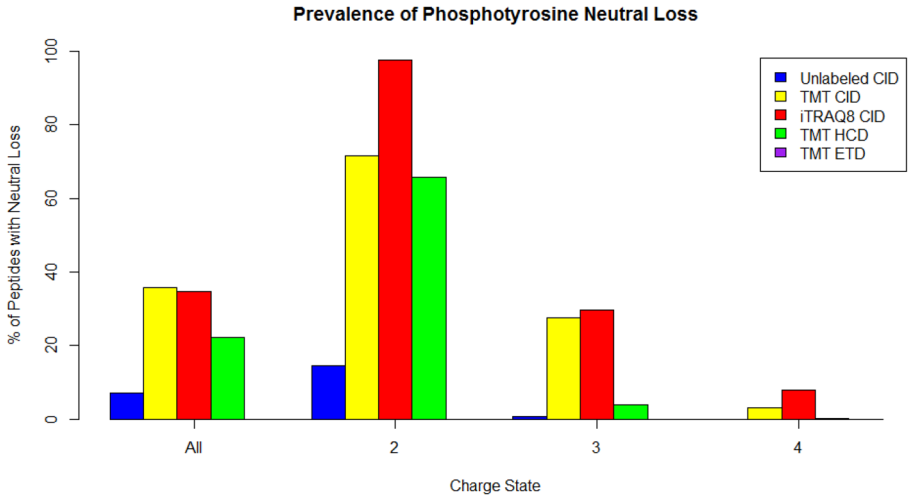

Figure 2.

Comparing the extent of neutral loss formation for five different conditions using the 2000 member phosphotyrosine library. A peptide was considered to have a neutral loss (NL) if the NL intensity was ≥ 33% of the base peak. Only ions within 0.02 Th of the expected NL masses were considered.

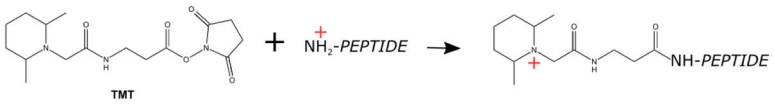

Figure 3.

The TMT labeling reaction is shown which results a primary amine being exchanged for a tertiary amine.

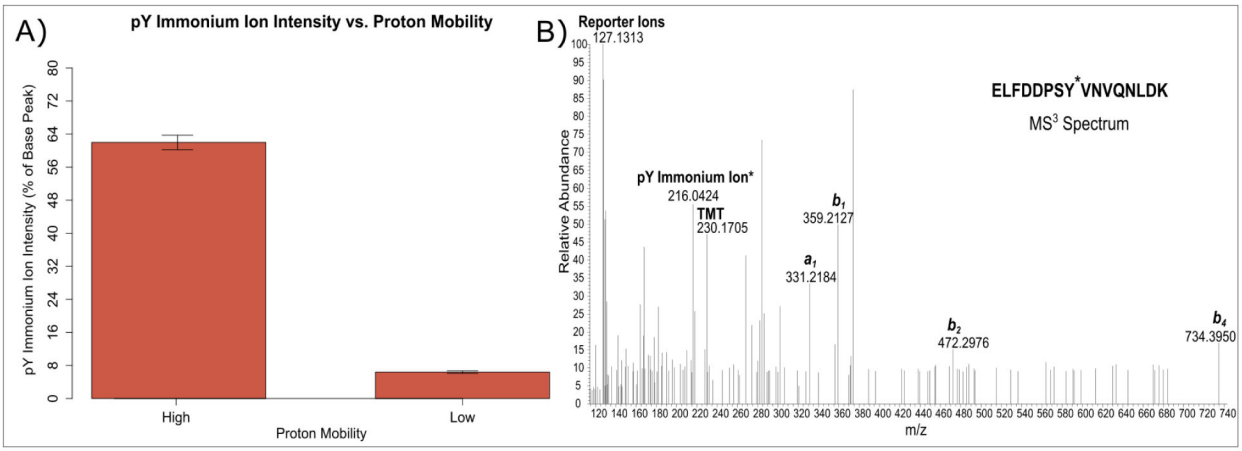

Figure 4.

(a) Comparison of the average phosphotyrosine immonium ion intensity (relative to the base peak) in terms of proton mobility, for all identified spectra from the 2000 member library. HCD fragmentation was used to fragment the TMT labeled peptides. The error bars represent ± the 95% confidence interval. The average pY immonium ion intensity was 10X higher if a mobile proton was present. (b) MS3 spectrum from a SHC1 peptide where the phosphorylation site was not localized to tyrosine by either the search or site localization algorithm based on information in the MS2 spectrum. The MS3 spectrum clearly shows the presence of the phosphotyrosine immonium ion, allowing localization of the site to tyrosine.