Identification of Tyrosine-Phosphorylated Peptides Using Cold Ion Spectroscopy

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S *Supporting Information*

ABSTRACT: The accurate and unambiguous detection of post-translational modifications in proteins and peptides remains a challenging task. We report here the use of cold ion spectroscopy for the identification of phosphorylated tyrosine residues in peptides. This approach employs the wavelength-specific UV fragmentation of cryogenically cooled protonated peptides in the gas phase. In addition to the appearance of specific photofragments, the phosphorylation of tyrosine induces large spectral shifts of the peptide electronic band origins. Quantum chemical calculations and experiments together suggest a certain generality of the use of such shifts in the spectroscopic identification of phosphotyrosines. The enhanced selectivity offered by the joint application of wavelength-specific fragmentation and mass spectrometry of cold molecules can also be used in the identifications of aromatic residues in protonated peptides and, potentially, of other UV-absorbing groups in a variety of large polyatomic ions.

Protein and peptide phosphorylation is the most abundant natural post-translational modification, which drastically increases the diversity of their biological functions. Abnormal phosphorylation in living organisms is associated with certain cell pathologies, and therefore the understanding and detection of this modification can be used for human disease diagnostics.[1,2] Methods of phosphorylation analysis include several biochemical, wet techniques, and high-resolution mass spectrometry (MS). Although MS in combination with collisionally induced dissociation (CID), high-energy collisional dissociation (HCD), and electron-capture/transfer dissociation (ECD/ETD) techniques can accurately identify the presence of phosphorylated peptides,[2,3] there are still many unresolved challenges facing the unambiguous determination of phosphorylation sites.[2] In particular, the most suitable approach for phosphoproteomics, ETD, is not applicable to singly protonated peptides. Here, we report the use of electronic spectroscopy of cryogenically cooled, singly protonated peptides for the detection of the spectroscopic signatures of their phosphorylation and suggest an approach for spectroscopic identification of peptides with phosphotyrosines.

Over the past few years, the gas-phase spectroscopy of biomolecules, when combined with high-level theoretical calculations, has demonstrated increasing power for the accurate determination of the intrinsic structures of oligopeptides.[4–8] Although this approach is, in principle, suitable for the assignment of phosphorylation sites,[5] its practical use is inhibited by unfeasibly time-consuming computations for large species. In certain cases the IR and UV spectra of cryogenically cooled peptides and their complexes can provide essential structural information without computations.[9] Protonated peptides are cooled to ~10 K[10,11] to sharpen their UV spectroscopic fingerprints, which allows assignment of Tyr phosphorylation without measuring the entire peptide spectra and without appealing to calculations.

Our experimental procedure and apparatus have been described in detail elsewhere.[10] In brief, protonated peptides are generated from water/methanol solutions using a nano-electrospray ion source. The ions of interest are preselected by a quadrupole mass filter and stored in an RF ion trap maintained at 6 K, where they are cooled via collisions with He gas. A pulsed, tunable UV laser induces the fragmentation of the trapped ions, and the fragments are ultimately detected by a quadrupole mass spectrometer (QMS).

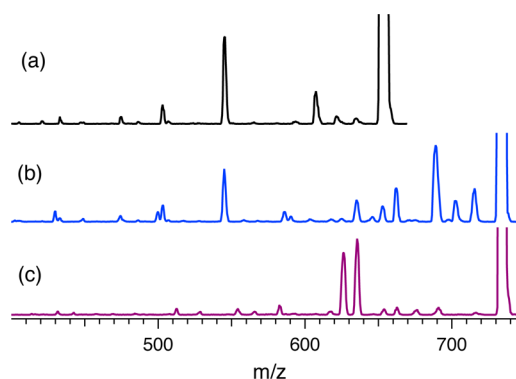Figure 1 compares the fragment mass spectra of singly protonated hexapeptide Ac-YA$_3$SK and two isobaric peptides,

Figure 1. Fragment mass spectra of singly protonated peptide (a) Ac-YA$_3$SK, (b) Ac-pYA$_3$SK, and (c) Ac-YA$_3$pSK, each recorded by isolating a precursor peptide in the cold ion trap and fixing the UV laser wavenumber at 35370, 37168.6 or 35137 cm^{-1} respectively. The truncated peaks at m/z values of (a) 653 and (b, c) 733 are due to precursor ions.

phosphorylated either on tyrosine or on serine. The spectra were measured with the UV laser wavenumber fixed at the electronic band origins of the ions (Figure 2). The most abundant photofragment (m/z = 545) of Ac-YA$_3$SK (Figure 1a) corresponds to the cleavage of the C$_\alpha$–C$_\beta$ bond with a loss
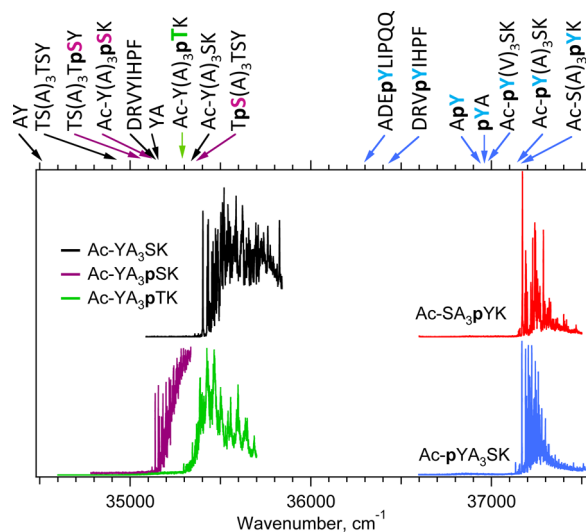
Figure 2. UV photofragmentation spectra of singly protonated peptide Ac-YA₃pSK and its phosphorylated modifications Ac-YA₃pSK, Ac-YA₃pTK, Ac-SA₃pYK, and Ac-pYA₃SK. The measured positions of UV band origins in all studied singly protonated peptides are shown above the spectra.

of the neutral Tyr side chain, Y_β. The UV photodissociation of the same peptide phosphorylated at tyrosine (pY) yields the same highly abundant charged fragment (Figure 1b), implying that the pY peptide loses a neutral phosphorylated side chain of tyrosine (pY_β). As could be expected, this characteristic fragment was not detected for the isobaric peptide phosphorylated on serine (Figure 1c). The prompt loss of neutral side chains in aromatic residues (W, Y, and F) is very specific to the UV excitation dissociation channel[12−15] and indicates the presence of the corresponding residues in peptides. The detection of the $[MH-pY_\beta]^+$ fragment unambiguously identifies the phosphorylation of the MH^+ precursor peptides on tyrosine.

The fragmentation yield of the side-chain loss channel varies significantly for different peptides, generally being lower for larger species.[12] This limits the sensitivity of the approach described above. A more sensitive, spectroscopic approach for the detection of phosphotyrosines relies on monitoring the appearance of any photofragments. Figure 2 presents the UV photodissociation spectra of $[Ac-YA_3SK + H]^+$ peptide and four of its phosphorylated modifications, cooled to ∼10 K. The spectra were measured by simultaneously detecting all prominent photofragments in the low-resolution mode of our QMS. One striking feature of these spectra is the very large ∼1770 cm⁻¹ blueshift of the electronic band origins in the two peptides phosphorylated on tyrosine. By contrast, phosphorylation on serine or threonine shifts the band origins only slightly and to the red. Spectroscopy of several other peptides demonstrates (top of Figure 2) that the band origins of pY peptides are always shifted strongly toward the blue with respect to the band origins of the peptides with non-phosphorylated tyrosine, leaving a spectral gap (∼35400−36200 cm⁻¹) between the two groups of band origins. This observation prompts us to suggest that large blueshifts might be intrinsic to the phosphotyrosine residue, such that the observed gap can serve for the identification of pY peptides. Indeed, phosphorylation on S and T residues can influence the chromophore absorption only through weak, noncovalent interactions, whereas in pY-peptides the phospho group

becomes covalently bound directly to the chromophore by replacing its hydroxyl hydrogen. Similar to the known cases of substituted aromatic molecules,[16] this replacement should reduce the conjugation of the π-electrons of the aromatic ring with the lone pair of electrons on the chromophore oxygen, leading to the observed blueshifts. Our quantum chemical calculations, which are still affordable for dipeptides, illustrate this mechanism for $[AY + H]^+$ and $[ApY + H]^+$ (see Supporting Information for details). Figure 3a shows the
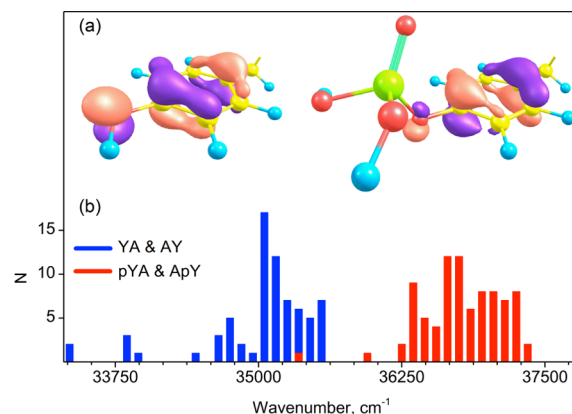

Figure 3. (a) Calculated HOMOs for AY (left-hand side) and ApY (right-hand side) singly protonated peptides. (b) The distribution of VTEs, calculated for 157 conformers of YA and AY protonated dipeptides and their phosphorylated analogs. The results are scaled by a factor of 0.87 to achieve the best match between the VTEs for the lowest-energy conformers of the two non-phosphopylated peptides and the measured positions of their band origins.

highest occupied molecular orbitals (HOMO) of the lowest-energy conformers calculated for these peptides. Upon phosphorylation, the orbital indeed becomes more localized on the ring, and the calculated (scaled) vertical transition energies (VTEs) yield a blueshift of 2700 cm⁻¹, which is close to the experimentally measured value of 2400 cm⁻¹ (Figure 2). The difference in the VTEs calculated for the isobaric dipeptide $[YA + H]^+$ and for its phosphorylated analogue is also in good agreement with the measured blueshift. This double validation provides us confidence in calculations of VTEs for higher energy conformers.

A comparison of the structures of the two pairs of peptides (Supplementary Figure S2) suggests that the difference in their blueshifts originates from the differences in the strong noncovalent interactions (proton−π interaction and hydrogen bonding) of tyrosyl with its local environment. For an arbitrary peptide, these contributions are difficult to account for because of peptide diversity. We have calculated VTEs for a total of 157 low-energy conformers of AY, YA, ApY, and pYA protonated dipeptides. Assuming that these conformers randomly mimic certain possible local interactions of the chromophore in larger peptides, we used the calculated VTEs to evaluate the likely statistics for the blueshifts of arbitrary pY peptides. The histogram in Figure 3b exhibits a large ∼500 cm⁻¹ spectral gap separating the distributions of the VTEs in phosphorylated and non-phosphorylated peptides. The probability for the VTEs of the two distributions to overlap or to fall into this gap is quite low (1.3%). This result is consistent with the experimentally observed spectral gap (Figure 2), suggesting that it should be present for a vast majority of peptides that contain either Y or pY.

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In contrast to the liquid phase,[18] the inhomogeneous broadenings in spectra of cold gas-phase molecules are much smaller than the observed spectral gap, enabling its reliable detection. On the basis of this observation, we suggest an approach for the purely spectroscopic detection of phosphotyrosine in peptides, using their wavelength-specific UV fragmentation. The diagram presented in Figure 4 summarizes
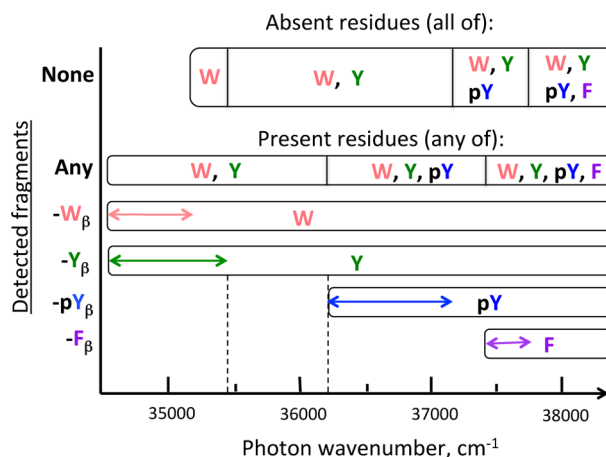

Figure 4. Diagram for the determination of the presence/absence of W, Y, pY, and F residues in aromatic peptides using wavenumber-specific UV fragmentation (two upper lines) or by detecting specific photofragments (four lower lines). The two dashed lines indicate the test window. The symbols $-W_\beta$, $-Y_\beta$, $-pY_\beta$, and $-F_\beta$ denote charged peptides that have lost their respective neutral aromatic side chains. The double-headed arrows indicate the approximate spectral intervals, where the UV band origins of the respective residues may occur. These data are taken from ref 13 (W), Figure 2 (Y, pY), and ref 17 (F).

the available experimental data regarding the positions of UV band origins in cold peptides. It correlates the presence/absence of the aromatic residues and pY in peptides with the wavenumber-specific absence/presence of any fragments and presence of specific fragments upon peptide photodissociation. There is a spectral region ($\sim$35400–36200 cm^{-1}) where W and Y residues always absorb, but the pY group never does. A lack of fragmentation at any single wavenumber within this "test window" therefore immediately implies the lack of W and Y in a peptide. If any fragments are then detected within the range of $\sim$37150–37400 cm^{-1}, then all tyrosines in the peptide are phosphorylated. Similarly, the absence of W but the presence of Y can be verified by subsequent observations of a lack of fragmentation within $\sim$35200–35400 cm^{-1} but the appearance of fragments within the test window. The fragmentation above $\sim$37750 cm^{-1} but a lack of it within $\sim$37200–37400 cm^{-1} indicates that F is the only aromatic residue present in a peptide. Alternatively, this purely spectroscopic approach for detecting chromophores should be complemented by the MS detection of specific (side-chain loss) photofragments, as described above. Figure 4 then can assist in the assignment of the suspected fragment mass peaks by correlating their appearance with the wavenumber of the dissociation laser. The diagram in Figure 4 does not yet consider many special cases (e.g., multiply phosphorylated peptides, sulfur bridges, metal ligands, etc.) and is based thus far on a limited number of observations. Nevertheless, it seeds a use of wavelength-specific UV fragmentation for gas-phase detection of phosphorylated tyrosine and aromatic residues in cold peptides. More generally,

such detections can be used as complementary constraints on MS determination of peptide compositions.

The large width of the test window in Figure 4 allows spectroscopic identification of pY residues at temperatures much higher than $\sim$10 K, as used in our experiments. Cooling pY peptides to only 100 K, for instance, would already greatly suppress the intensity of their UV hot bands (<3% of the main band) in the middle of this window, although the gap would be completely blurred at room temperature.

The UV spectra presented in Figure 2 provide no apparent evidence of phosphorylation on Ser or on Thr. In light of this observation, we attempted to find specific absorptions of phosphopeptides associated with phosphate OH-stretch vibrations in 3 μm region (Supplementary Figure S5). In contrast to an earlier report,[19] where in a few small peptides these absorptions appeared above 3650 cm^{-1}, we did not detect any absorption in this region for the larger peptides, phosphorylated on S and T. This makes ambiguous a use of mid-IR spectroscopy for the detection of peptides phosphorylated, at least, on these two residues. The fundamental reason for this ambiguity is likely the high probability for hydroxyl groups to form hydrogen bonds, which may redshift and broaden the OH-stretch absorption bands.

In conclusion, we have demonstrated the use of cold ion spectroscopy for the gas-phase identification of phosphotyrosines and aromatic residues in protonated peptide. The wavelength-specific UV excitation of such peptides produces specific charged fragments as a mass spectrometric signature of pY, as well as W, Y, and F residues. A more sensitive, spectroscopic approach for the detection of peptides, in which all tyrosines are phosphorylated, relies on the large blueshifts of the UV band origins that are induced by phosphorylation. Our measurements and calculations together suggest that this identification approach should be highly reliable. Although explored here for peptides only, the high selectivity offered by the combination of cold ion UV spectroscopy and mass spectrometry has a high potential for the use in the computation-free identifications of UV absorbing groups in a variety of large polyatomic ions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Details and results of the calculations for conformers of the protonated dipeptides; IR spectra of the protonated hexapeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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